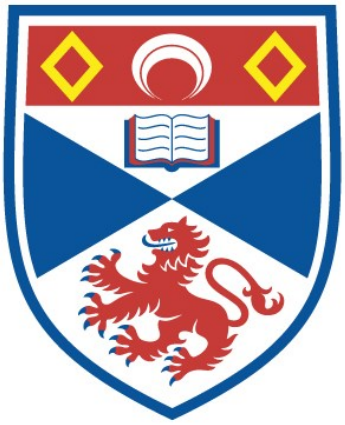
A STUDY OF THE COURTSHIP SONG PARAMETER IN THE 'DROSOPHILA MELANOGASTER' SPECIES COMPLEX

Adrian R. G. Pugh

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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I would like to dedicate this to Emma Damoglou, my wife. Sincerest of thanks must be reserved for her unfailing love, support, and trust in me throughout our entire time together. She has repaid the meagre support I have given her a thousand fold. Most of all she has blessed me with our daughter. No words of gratitude would be enough to express the love I have for both of them.

DECLARATION

I, Adrian R. G. Pugh, hereby certify that this thesis, which is approximately 40,000 words in length, has been composed by me, and that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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I was admitted as a research student in October 1993 and as a candidate for the degree of Ph.D. in October 1993; the higher study for which this is a record was carried out in the Faculty of Science of the University of St. Andrews between 1993 and 1997.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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Thanks of course to Emma for everything. Of course, the arrival of my daughter Kathryn means I now have two females in my life. I freely forgive the resultant delay of my write-up Katy caused, and thank her for not being sick on my keyboard. My parents have supported me from her age, and I am always grateful. Thanks also to my sister Janet for having to suffer a younger brother. A special thanks also goes to Michelle for her hours of baby-sitting while a finished this beast off.

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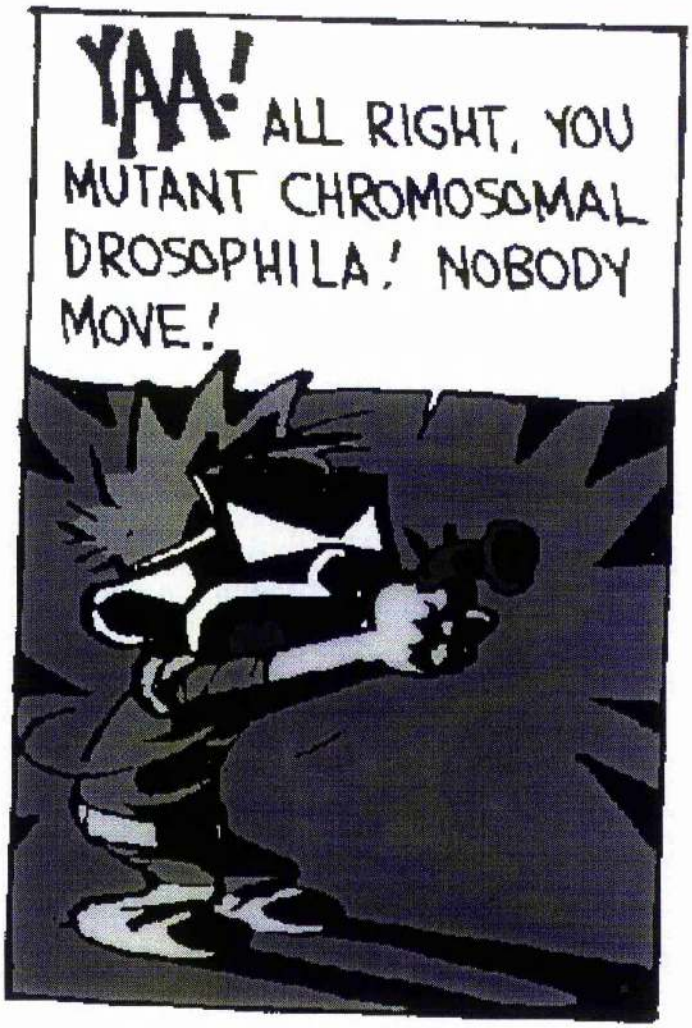
To all those who stay in the Old Course Hotel I would just like to say 'Get your own coffee you lazy slobs!'



Just another day in the lab

"... Male fruit flies are famous for their sense of romance. They begin the mating ritual by 'singing' a ... love song - with their wings - to their beloved during an enchanting fly pass."

Tim Walker - "What has the fruit fly done to deserve this?" Daily Mail, June 6 1995

"If music be the food of love, play on"

William Shakespeare - "Twelfth Night"

ABSTRACT

Sexual isolation between populations has long been thought to play a role in speciation and evolution. However, the genetic control of factors causing sexual isolation is still not clearly understood.

The interpulse interval (IPI) of the pulse song of the *Drosophila melanogaster* complex is important in female preference and species recognition. This thesis examined the genetic control of IPI. IPI may be important in the sexual isolation of species within the *D. melanogaster* complex, notably the three species *D. melanogaster, D. simulans,* and *D. mauritiana.*

The genetic control of differences in IPI between *D. simulans* and *D. mauritiana* was studied using backcrosses and marker loci. The results showed that the control of the difference in IPI between species was evenly spread across five equally sized regions of the genome.

Bi-directional artificial selection for shorter and longer IPI was carried out using replicate lines to control for genetic drift. The difference between selection regimes achieved was over 4.5 msecs. The difference was fixed using chromosome balancers to prevent recombination. Mating speeds of females from the selection lines with males from the selected and unselected lines were not significantly different from the original unselected stock following selection.

The mean IPI of an African strain of *D. melanogaster* that had previously been shown to demonstrate premating isolation from other strains of *D. melanogaster* was measured, and was found to be significantly shorter than the control strain. Female mating speed of the African strain with males of the same strain was significantly faster than mating speeds with males of two strains with differing distributions for IPI.

Heritability and evolvability of two song traits including IPI, and four morphological traits were measured in two generations of a strain recently derived from a wild population. All values were low, and mostly non-significant. The values for IPI were compared to values obtained from laboratory stocks, and with the values obtained for morphological traits.

This study has furthered the understanding of the genetic control of IPI, and female response to changes in IPI. The examination of differences in IPI between populations and species remains important in our understanding of traits affecting sexual selection and premating isolation.

CHAPTER 1: INTRODUCTION

1.1 Species and speciation

1.1.1 Defining speciation and reproductive isolation

Ernst Mayr (1942) wrote the most widely used definition of species: "Species are groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups." This one sentence aims to describe the continuity of genetic traits for individuals within a species, and the discontinuous genetic nature of individuals of different species. However, this simple definition has been hard to equate with the wide spectrum of isolation between populations in nature. The problem with any evolutionary definition of a species is that it attempts to define two basic aspects. The first being what makes up the differences between species, and/or the nature of individuals that should be included together as one species. The second is the process of speciation itself. This process, cladogenesis (the splitting of lineages), can be a gradual one over time, involving many changes that contribute to the resulting isolation.

The effects of morphological, behavioural and temporal isolation interact with the isolation between species caused by physiological incompatibility factors such as sperm sterility. For example, crosses of *Drosophila simulans* males with *D. sechellia* females can result in fertile female, and sterile male hybrids, but reciprocal matings are rare (Lachaise *et al.*, 1986; Cobb and Jallon, 1990). This example demonstrates how behavioural elements can profoundly affect reproductive isolation even when hybridisation is possible. In such species several distinct factors result in the isolation of populations in nature that lead us to call them separate species.

Barriers to gene exchange are split up in to two broad groups; premating and postmating isolation. The reasons for the occurrence of each type is described in table 1.1.

PREMATING ISOLATION	POSTMATING ISOLATION
Not present in the same locality	Fertilization does not take place
Sexually active at different times	Abnormal development of the zygote
Lack of recognition of each other as potential mates	Mortality of hybrids before maturity
Lack of stimulation of sexual receptivity in each other	Hybrids fail to obtain mates
Failure to achieve intromission (internal fertilization), or co-ordinate gamete release (external fertilization).	Hybrids are infertile

Table 1.1 Causes of premating and post mating isolation.

Geographic barriers between populations allow physical separation during which time genetic differences between these populations can accrue in the absence of genetic exchange between the populations. However the genetic basis of any changes that cause reproductive isolation is far from being fully understood.

Coyne and Orr (1989, 1997) showed that both mating discrimination, and sterility and inviability of hybrids between species of the genus *Drosophila* accumulated steadily with genetic distance and hence time. Allopatric species have comparable rates of evolution for discrimination and sterility, but in sympatric species discrimination appeared well before strong sterility or inviability. This may be due to strong reinforcement of prezygotic isolation when formerly allopatric taxa meet, combined with the fact that taxa without a sufficiently strong premating isolation would merge upon re-encounter.

Most work on speciation genetics has concentrated on post-mating factors due to their ease of measurement, and because these are mainly used as criteria for defining species. The role that premating isolation plays has been under-emphasised

despite it's relative importance. There is a resulting bias in the number of reported post-mating factors over pre-mating factors which is due to the number of studies reported. The bias is therefore an artefact, and not a reflection of the inherent in speciation.

Paterson (1985) has formed an alternative definition to the classical biological species concept of Mayr. His "species recognition concept" is derived from the mating systems of the animals. He claims that physiological barriers are irrelevant, and that it is the isolation caused by mating discrimination that is the only factor responsible for the separation of two populations into distinct species. This is widely thought of as being inaccurate as it totally ignores sterility and inviability. It does highlight the importance sexual isolation can play in causing the isolation necessary between populations to prevent genetic interchange even without any postmating factors involved.

1.1.2. The relative contribution of genes of major and minor effect on sexual isolation and speciation

A question of much interest is whether change at one or more genes is necessary for speciation to occur. The classical theories of geographic speciation suggest a more gradual accumulation of mutations in many alleles of small effect (Charlesworth, Lande & Slatkin, 1982). Some work has supported this by showing how factors causing isolation are controlled by several regions of the genome (Coyne, 1983, 1984, 1985a, 1993). However it is impossible to say which of the gene substitutions causing the difference between the two species occurred during speciation (and are thus responsible for the isolation), and which occurred after, thereby reinforcing the pre-existing isolation.

Templeton (1981, 1982) suggested that speciation involving small (island) populations would result in differences between these populations due to a few allelic

substitutions of large effect. Wright (1982) points out that simple genetic changes causing major character differences would be associated with the occupation of a new niche. Gould (1977) and Stanley (1979), have also suggested that single mutations of large effect are important in speciation and macroevolution.

The theory of punctuated equilibrium, and Carson's theory of founder event speciation (1975), also predict that most of the change resulting in genetic differences between species arise primarily during the speciation event with a lower subsequent rate of change. However some morphological data would seem to contradict this (Coyne, 1985a; Coyne and Orr, 1989a).

It has been suggested that for it's size, the X-chromosome plays a disproportionate role in speciation (Coyne and Orr, 1989). Members of the heterogametic sex have only one copy of the X-chromosome. Any X-linked mutation in the heterogametic sex will, when recessive and [illegible] more than an autosomal counterpart.

In sexual selection, one sex, commonly the male, will display a morphology or behaviour, which is called a "trait". The trait can vary between individuals the population. One other sex (usually females) will then display a preference for individuals possessing certain levels of the trait. These levels will differ between the population. The strength of the individual's preference will be the selection pressure with which the trait is selected. Genes for a trait selected by a shift in the male trait could cause a similar shift in female preference. A more stable linkage between these genes could allow stronger selection. The X chromosome has reduced recombination in *Drosophila* therefore linkage associations on the chromosome might remain intact longer than on the autosomes.

Orr and Coyne (1992) looked at the evidence of major genes in species differences. They used three criteria when looking at the genetics of species differences;

a) An analysis that could distinguish between alleles of large and small effect.
b) A clearly adaptive interspecific difference.
c) The difference was seen in nature and not due to artificial selection (e.g. cultivars).

Orr and Coyne found the evidence for polygenic genetic control of speciation was surprisingly scarce in studies meeting these three criteria. Before the advent of molecular markers experiments were highly limited. There were relatively low numbers of markers available for a small number of species. Also relatively few studies met the criteria. The use of quantitative trait loci (QTL) analysis uses molecular markers to allow more detailed studies than is possible with phenotypic markers alone. In recent studies (e.g. Bradshaw *et al.*, 1996; Liu, 1996) this form of analysis has allowed more resolution of the relative contribution of areas of the genome. It has also expanded the range of species that can be studied. Studies have previously been restricted to a few species, that are usually laboratory based. The results obtained seem to support the notion that a few genes can play a significant role.

1.1.3. Mate recognition, and species recognition.

The prevailing approach of previous studies of animal communication signals such as *Drosophila* song is to either use the signal to study species recognition or sexual selection. The separation into two groups is mainly due to studies being limited to differences within a species, or between species, but rarely both. The result of this separation has led to an artificial separation of the variation in female responses, which has been split into examinations of response to either conspecific males, or between conspecific and heterospecific males.

which has been split into examinations of response to either conspecific males, or between conspecific and heterospecific males.

Mate recognition has been defined as "a behavioural response to another individual, as if the subject thinks that individual an appropriate mate" (Paterson, 1985). The definition does not imply any comparison and/or preference between potential mates producing a signal. Therefore females that recognise a male from a different species, but show a preference for one of their own species, are discriminating in exactly the same manner as if they were showing sexual preference between two males from the same species. Thus species recognition can be defined as the "sexual preference for mates displaying a conspecific type call over that of one displaying a heterospecific type call, when both signals elicit a response" (Paterson, 1985).

These definitions assume that the range of the signal of the conspecific is different from the range of the heterospecific and is coincident with the range in which female preference occurs, and is at its highest. This may not always be the case. Ryan and Rand (1993) studied the mating call of frogs in the *Physalaemus pustulosus* species group, and found that while female *Physalaemus pustulosus* always preferred conspecific calls, they preferred conspecific calls with the prefix of a heterospecific members of the group. Therefore the conspecific call does not occur at the peak of the female preference curve. They then described five possible interactions between male trait and female preference for one species, as shown in the figure 1.1 below.

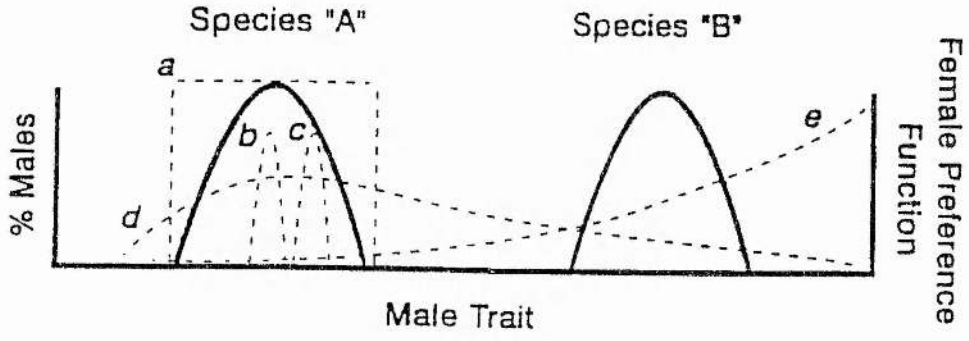


Figure 1.1 Preference functions of females (*a, b, c, d, e*) from species A and B, and the mating signals of males from both species (A and B). For full description see text.

Preference functions *a* to *d* all show preference for conspecific signal over a heterospecific signal. Preference function *a* to *c* show no response to the heterospecific signal. Preference function *a* does not generate selection, and *b* and *c* generate stabilising and directional selection respectively. Lastly in preference function *e,* both species elicits a response; however the heterospecific signal is preferred. This is also used as an example of females preferring a "supernormal" signal that may not be present in the range of the conspecific males.

In studies of sexual selection within a species, it is presumed that female preference is for an extreme signal, and that the male's signal has evolved to try to match female preference. If there is some extrinsic barrier to the convergence of signal and preference then it may be possible for a heterospecific male to possess a signal which is more preferred by that individual (as in preference function *e*). In the genus *Xiphophorus,* female platyfish prefer males with swords appended to their tails, a trait that is absent in platys but present in congeneric swordtails (Basolo, 1990). *X. pygaeus* prefer the larger heterospecific males from *X. nigrensis* which display a courtship display that is absent in conspecific males (Ryan and Wagner, 1987).

In a founder event, parental females with a preference function similar to *b* will produce offspring whose range of preferences can increase due to the lack of selective pressures limiting the preference range. This will give rise to a preference function similar to *d* (as suggested by Kaneshiro, 1976), and other selection pressures could cause a shift in male trait. If the shift is not countered by the pressure of sexual selection, and female preference is bimodal, the male trait could be shifted before re-establishment of strong female preference.

Sexual preference may be outwith the range seen in the trait within the population. However the male signal had been constrained by other selective pressures from converging with the preference peak e.g. predation risk for males with a conspicuous signal. The countering effects of natural selection such as predation would indirectly act against females who selected for males with more conspicuous

signals, stabilizing the distribution of female preference. A founding population might be established in a habitat where these predation constraints were lessened or absent, allowing the male trait to converge with female preference. Female guppies, *Poecilia reticulata*, prefer males with more orange skin pigment, and the female preference varies between and within populations (Houde, 1987; 1988). This pigment makes them more conspicuous to predators and it has been shown that females in areas with lower predation risk preferred more brightly coloured males than females in areas of high predation risk (Stoner and Breden, 1988, Houde and Endler, 1990).

Ryan and Rand (1995) used reconstructed estimates of calls from ancestral nodes in *Physalaemus pustulosus*, and examined female response. Females showed no discrimination between the conspecific calls and the reconstructed call from the most recent node. They conclude that the evolution of the song was not in response to female preference but to other selective pressures on the call. This also suggests uneven and uncoupled evolution of the signal and receiver systems.

1.2. The *Drosophila melanogaster* species subgroup

1.2.1 Historical Biogeography of the subgroup

The Melanogaster species group consists of about 150 species and is split into eleven species subgroups. The Melanogaster subgroup is the most extensively studied of these subgroups. It contains eight species that evolved from a common ancestor of oriental origin during the Pleistocene some 1.7-2 million years ago. The taxonomic relationship is show in figure 1.2 below.

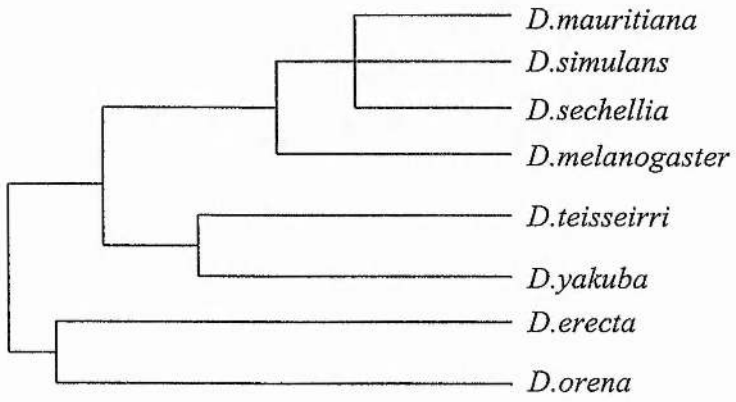


Figure 1.2 Phenogram showing the species relationships within the Melanogaster complex based on a review of possible phenograms by Lachaise *et al.*(1988).

The first divergence lead to a split of the erecta-orena ancestor from the rest of the subgroup followed by the divergence of the teissieri-yakuba ancestor from that of the Melanogaster complex. This complex has radiated into four species with an initial split of *D. melanogaster* about 1.5 million years ago, followed by the divergence of *D. simulans, D. sechellia* and *D. mauritiana*. The exact nature of the latter divergence is unknown, being either due to separate divergences of *D. sechellia* and *D. mauritiana* from *D. simulans*, or successive divergences of *D. mauritiana* from *D. simulans*

from *D. simulans*, or successive divergences of *D. mauritiana* from *D. simulans* followed by that of *D. sechellia* from *D. mauritiana* (Lemeunier *et al.*, 1986). Two studies by Hey and Klimen claimed that the results did not support *D. sechellia* and *D. mauritiana* as being the most closely related species, although they were unclear on the relative timing of the divergence (Klimen and Hey, 1993; Hey and Klimen, 1993). A more exhaustive study of the phylogeny using molecular polymorphisms contradicted Hey and Klimen's conclusion, placing *D. mauritiana* and *D. sechellia* most closely together, having diverged from a common ancestor with *D. simulans.* (Caccone *et al.*, 1996).

D. erecta is a rare species found most abundantly in the Ivory Coast area. The species has a close association with the screwpine *Pandanus*, which occurs in swampy and stream-side areas. Population density of *D. erecta* is high and strictly dependent on the *Pandanus* fruiting season. It is low outwith the *Pandanus* season, with flies showing more opportunistic behaviour in food sources.

D. orena is thought to be extremely rare, and is known only from ten wild caught males and one female found in West Cameroon, from which all lab stocks have been founded. Difficulty to breed in the lab suggests a specialist nature.

D. teissieri and *D. yakuba* occupy similar ranges to each other in Eastern Africa. There is a rough east-west divide; *D. teissieri* is the more western. The border seems to be due to an ecological divergence: *D. teissieri* is chiefly a forest species, and *D. yakuba* is chiefly an open field species. Although the two species are often found together, their relative abundance is dictated by the habitat.

D. melanogaster is a cosmopolitan species originating from Western Equatorial Africa, and is the most well studied of the subgroup. Populations seem to be associated with human activity, but museum material suggests that there may be an association with *Lobelia* as a host plant. *D. simulans,* also cosmopolitan, originates from Eastern Equatorial Africa. It is virtually absent west of the Cameroon mountains. The two species are thought to have been totally disjunct in recent historical time.

Both have spread, with the aid of human beings, to Asia, Europe, America, and Australasia (Lachaise *et al.* 1988; Lemeunier *et al.* 1986).

D. sechellia and *D. mauritiana* are homosequential with *D. simulans,* and are insular endemics found only in the islands of the Seychelles and Mauritius respectively. *D. sechellia* has a strict association with the fruits of the rubiaceous shrub *Morinda citrifolia. D. mauritiana* is an abundant broad niched, opportunistic species, despite its restricted home range. *Morinda* may have been its original host plant before the arrival of human beings to the islands (David *et al.*, 1989).

Although some *D. melanogaster* occur in *D. mauritiana's* natural range the former species tends to be limited to harbour warehouses and areas of human activity. In these areas natural sterile female hybrids have been found. *D. simulans* does occur on the Seychelle and Mascarene islands but not on those islands where *D. sechellia* and *D. mauritiana* occur, and therefore in the Indian Ocean the three species occur in allopatric insular populations.

1.2.2. Interspecific crosses within the Subgroup

Crosses between the species *D. yakuba, D. teissieri, D. erecta*, and *D. orena,* produce no viable hybrids. Crosses of *D. simulans, D. mauritiana*, *D. sechellia* with *D. melanogaster* produce sterile hybrids of the sex of the *D. melanogaster* parent and inviable hybrids of the other sex. Mutations are known that can rescue the lethality in the other sex. Recently a strain of *D. simulans* has been found that can produce fertile, viable F1 female hybrid offspring when mated to *D. melanogaster* (Davis *et al.*, 1996).

All crosses between *D. simulans, D. mauritiana*, and *D. sechellia* produce viable F1 hybrids of both sexes, with the females being fertile and the males sterile. These crosses follow Haldane's rule, which states that when the offspring of one sex from the cross between two different taxa are absent, rare, or sterile, it is the

heterogametic sex" (Haldane, 1922). Genetic backcross study between *D. simulans* and *D. mauritiana*, or *D. sechellia* is possible by using the fertile female F1 hybrids and backcrossing these to the parents in most cases. In addition *D. mauritiana* males produce sterile, viable hybrids of both sexes when crossed with *D. yakuba* or *D. teissieri* females, and only sterile viable females when crossed with *D. erecta* females.

Male \ Female	D. melanogaster	D. simulans	D. mauritiana	D. sechellia
D. melanogaster	-	Sterile | 0	Sterile | 0	Sterile | 0
D. simulans	0 | Sterile	-	Sterile | Fertile	Sterile | Fertile
D. mauritiana	0 | Sterile	Sterile | Fertile	-	Sterile | Fertile
D. sechellia	0 | Sterile	Sterile | Fertile	Sterile | Fertile	-

Table 1.2 Interspecific crosses within the *D. melanogaster* complex. Crosses in rows have the same male parental species, and crosses in columns have the same female parental species. Hybrids are listed male first (male | female). 0 = no viable offspring

1.2.3 The genetic basis of postzygotic isolation between species in the *D. melanogaster* complex

The genetic control of postzygotic isolation within the *D. melanogaster* complex is the result of several differences between species. The genetic control of the differences has been studied to establish the relationship of the species in the complex as well as the genetic causes of Haldane's rule. The studies use backcross techniques to compare the relative effects of factors causing sterility in hybrids with varying amounts of genome from each species, and also the effects of different regions of the genome, particularly the X-chromosome, on hybrid sterility. The usual method of identifying the genetic basis of traits with phenotypic markers is to analyse the segregation of the trait in hybrid and backcross progeny e.g. Coyne (1984). However

genetic analysis of courtship song parameter, interpulse interval (IPI) within the melanogaster subgroup is limited by the absence or sterility of the F1 hybrids with crosses involving *D. melanogaster*.

Coyne (1984) showed at least five loci were responsible for male sterility in backcross males between *D. simulans* and *D. mauritiana,* and that the X-linked component played a disproportionately large role. Coyne and Charlesworth (1986) have mapped this X-linked component. Davis and Wu (1996) studied the X-linked factors affecting sterility using visible and DNA markers. They identified three *D. mauritiana* components, one of which was itself comprised of three components. Difference in ovariole number in hybrids, affecting the number of potential offspring, between *D. sechellia* and *D. mauritiana* has been shown to be due to at least two substitutions, one on each autosome (Coyne *et al.*, 1991a).

Zeng and Singh (1993a) found that by introgressing the Y chromosome from one species into the genetic background from a different species between crosses of *D. simulans*, *D. sechellia* and *D. mauritiana* there was no effect of X-Y or X-cytoplasm interactions on male hybrid sterility. The result indicates that an X-autosome interaction is responsible for sterility involving the sex chromosomes.

Zeng and Singh (1993b) studied protein divergence between *D. simulans* and *D. sechellia*. They found a few *D. sechellia*-specific proteins associated with male sterility, suggesting relatively few genes involved in hybrid sterility, and that the disproportionately large effect of the X-chromosome was not due to higher divergence of the X-chromosome.

Postzygotic isolation within the *D. melanogaster* complex is usually due to mutations of large effect invariably located on the X-chromosome. However, as the regions of major effect are studied with greater resolution, they show several loci within the regions all contributing to the overall affect. Close linkage of several loci on the X chromosome would allow a high heritability of these isolating factors.

Postmating isolation is often due to mutations of large effect on the X-chromosome in other *Drosophila* species. Orr (1987) studied hybrid sterility between

D. pseudoobscura and *D. persimilis*, and showed that the lack of production of motile sperm was due to an incompatibility between the X and Y chromosomes. Male hybrid fertility was affected by a total of at least nine loci (five X-linked, one Y-linked and at least three autosomal). The semi-sterility in the females was due to an X chromosome-cytoplasm interaction. One notable exception of the involvement of the X-chromosome in isolation is male sterility between *D. texana* and *D. virilis* which has been shown to be due to Y/autosome interactions (Lamnissou et at, 1996).

The magnitude of effect of the X-chromosome has recently been questioned. Hollocher and Wu (1996) found a strong effect of homozygous, as opposed to heterozygous introgression of regions from the second chromosome from *D. mauritiana* and *D. sechellia* into *D. simulans*. They did not, however, find evidence of strong X-linked bias in the evolution of hybrid sterility, but did find strong bias in the evolution of hybrid sterility. They point out that the magnitude of the autosomal effect will be underestimated in backcross experiments, as only one of the two copies of the autosome is replaced. Recessive mutations on the autosomes from one species will not be expressed, but similar mutations on the X-chromosome will.

1.3 Courtship within the Subgroup

1.3.1 The structure of courtship.

The exact composition of courtship for each species within the subgroup varies, but there are several aspects that occur to a greater or lesser degree in all species. Aspects of mating are categorised into eight male and five female behaviour elements shown in table 1.3 (After Welbergen, 1991).

Male elements	Female elements
I. Orienting towards a standing female	I. Extrusion
II. Following a moving female	II. Kicking
III.Circling the female	III.Flicking the wings
IV.Vibrating one or both wings	IV.Decamping
V. Scissoring of the wing (not *D. erecta* and *D. yakuba* (Cowling and Burnet, 1981))	V. Standing
VI.Licking or proboscis extension	
VII.Attempted copulations	
VIII.Standing	

Table 1.3 Courtship behaviour elements of the *melanogaster* subgroup. (After Welbergen, 1991).

Within the *melanogaster* subgroup, Cobb *et al.* (1987) found a negative correlation between courtship latency and locomotor activity, so females slow down in order to allow males to mate. Overall for females, courtship latency decreases between one and two days after eclosion and then increases between five and six days. Time of day was not shown to play a role in courtship latency (Cobb *et al.*, 1989).

1.3.2 The genetic basis of prezygotic isolation between species in the *D. melanogaster* complex

Sex combs are thought to play an important role in the ability of male *D. mauritiana* to inseminate females. Coyne (1985a) found that difference in sex comb tooth number between *D. simulans* and *D. mauritiana* involved at least five alleles, with equal effects of the two autosomes and a smaller effect of the X-chromosome. This is not so important for *D. simulans*.

Coyne *et al.* (1991b) showed that both autosomes played a role in differences in male genital arch morphology between *D. sechellia* and *D. mauritiana*, with no significant effect of the X-chromosome. Liu *et al.* (1996) used QTL analysis and found evidence of genes of major effect between *D. simulans* and *D. mauritiana* controlling differences in genital arch morphology. Although there was some epistasis, inheritance was mainly additive.

There is an abnormally short mating duration between *D. simulans* and *D. mauritiana*. There might be female discrimination based on male genitalia. The interruption of sperm transfer associated with these short matings causes reproductive isolation when compared to the longer intraspecific matings. Coyne (1993) found that short mating time is influenced by the male parental genotype more than the female, with the *D. simulans* genes being dominant. This is due to a minimum of three loci. The two autosomes have the largest effect.

1.3.3 The role of cuticular hydrocarbons in *D. melanogaster* courtship

The role of cuticular hydrocarbons, or contact pheromones, in eliciting and inhibiting courtship has been found to be important. The male pheromones act to decrease female activity which allows males an opportunity to mount the female. Female olfactory mutants do not show decrease in activity, as seen in wild-type flies, which decreases the probability of successful copulation (Gailey *et al.*, 1986). 7-tricosene (7-T) is the predominant hydrocarbon in males of all species of the *melanogaster* subgroup except *D. sechellia* and some strains of *D. simulans* (Jallon and David, 1987).

Drosophila melanogaster displays sexual dimorphism for cuticular hydrocarbons. Males possess the compounds 7-tricosene and/or 7-pentacosene (7-P) depending upon the strain. These are virtually absent in virgin females, are acquired during mating by contact with males; and these two compounds have been shown to function as antiaphrodisiacs to the males (Scott, 1986). In the Canton-S strain, in which 7-T is predominant, females mate more readily with males from the same strain than with males from strains where 7-T is absent, or where 7-P is predominant (Jallon, 1984).

Scott and Richmond (1988) showed that production of hydrocarbons is controlled by both X-linked and autosomal loci. The X-linked loci acts as an enhancer for the production of the predominant hydrocarbon, and autosomal factors control a high versus low production of each of the hydrocarbons. There is significant negative correlation between the quantities of 7-T and 7-P in the F2 generation. The correlation indicates autosomal linkage between loci regulating expression of each. Additional unlinked loci are involved in production of each pheromone. Ferveur and Jallon (1996) examined the control of relative production and found that the second chromosome controls the change in balance of both pheromones, and the third chromosome regulates the overall quantities of both.

Scott (1994) found that females from the Canton-S strain discriminated between Canton-S and Tai-Y males, but Tai-Y females did not. While Canton-S males have 7-T as the predominant hydrocarbon it is virtually absent in Tai-Y males. The variation in female discrimination was shown to be based on chemical signals from the male hydrocarbons, and was controlled by a gene or genes on chromosome three.

As the Canton-S phenotype is conserved thoughout most of species of the *melanogaster* subgroup, the loss of (predominance of) 7-T has been accompanied by the loss of discrimination for it by females and is likely to be the derived state. The relative order of evolution of pheromone production and discrimination is unclear.

Most populations of *D.* simulans display 7-T as the predominant hydrocarbon. However some populations show a low ratio of 7-T to 7-P, and the polymorphism maps to one locus (Ngbo) on the second chromosome, showing additive expression of the two alleles, with co-dominant high/low expression of 7-P, with some secondary effects on 7-T production (Ferveur, 1991).

D. sechellia unlike the other species of the *D. melanogaster* subgroup have 6-T as the predominant male pheromone instead of 7-T. Each of the five main chromosomal regions has at least one gene involved in controlling the relative ratios of 6-T and 7-T, with the right arm of the third chromosome having the largest effect. The effects are therefore polygenic and generally additive, with some epistasis among third chromosome genes. Although some slight degree of sexual isolation was seen in hybridizations using males coated with heterospecific hydrocarbons, this is small compared to the role of female pheromones (Coyne, 1996b).

Although work on hydrocarbons has concentrated on variation of hydrocarbons in males, there are some studies of the variation in females. *D. melanogaster* females of most populations have high levels of 7,11 heptacosadiene (HD). A study of natural populations found females from Sub-Saharan Africa and the Caribbean that were unique in having low levels of 7,11 HD and high levels of 5,9 HD. This mapped to chromosome three. Females from strains with high levels of 7,11 HD mated more rapidly than those with low levels of 7,11 HD (Ferveur *et al.*, 1996).

In a backcross experiment, Coyne *et al.* (1994) showed that the sexual isolation which had previously been demonstrated between *D. simulans* males and *D. sechellia* females was due to the ratio of the hydrocarbons 7-T and 7, 11-HD. Differences in the ratio of the hydrocarbons of backcrossed females was mapped to chromosome three, suggesting a relatively simple genetic control. It is not known if the gene(s) controlling discrimination within and between species are the same, and any link is speculative at this time. 7, 11-HD is likely to act as an inhibitor of male courtship in *D. simulans*.

1.3.4 Physiology and the role of wing vibration in males, and sound reception in females.

Male wing vibration during courtship has been shown to produce an acoustic signal that can be categorised into two types of sound (Shorey, 1962; Von Schilcher, 1976a). Sine or hum song consists of a modified sine wave which is important in stimulating the female and increasing receptivity to male courtship . The other song type is called pulse song, which consists of bursts of discrete pulses. One day old virgin females show reduced locomotor activity in response to pulse and sine song, which was not seen with just pulse song alone (Schilcher 1976a, 1976b). Sine song occurs at periods throughout the song, and even during pulse song wing vibration. Figure 1.3 shows a trace of both these song types.

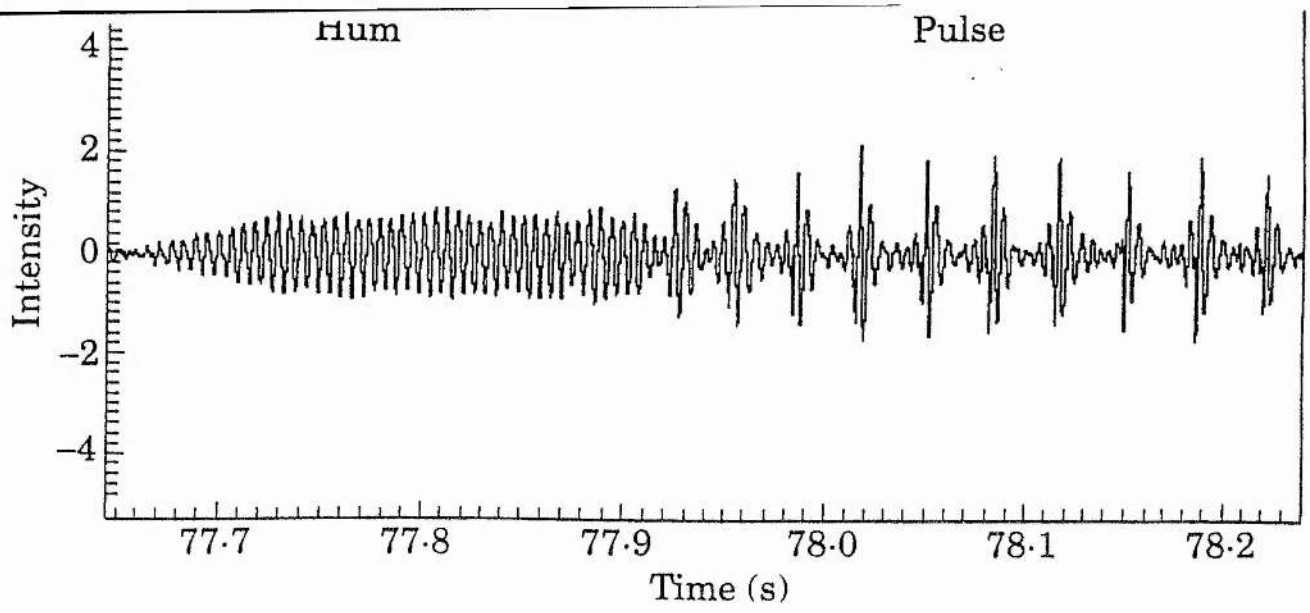


Figure 1.3 A trace of a *Drosophila melanogaster* song showing sine (hum) and pulse song.

Pulse song is generated by the vibration of the wings of the male by the contraction of the flight muscles in the thorax. The physiology involved in fly wing contractions is the same for flight and song production. The elastic potential generated by the slower downbeat of the wing is released causing the wing to 'bounce back' with a faster upbeat to the elevated position, with the cycle then being repeated. The down beat is more constant throughout the stroke, with the upbeat starting slowly then speeding up. Bennet-Clarke and Ewing (1968) found that cutting one of the dorso-ventral flight muscles stopped courtship vibration as well as the initiation of fight and jumping. In the courtship display one wing is extended to the side to roughly 90^{o}. The wing is initially raised slightly then the downstroke occurs. The air particle displacement causing the "noise" of the pulse is produced with the downbeat.

The pulse is sensed by the aristae on the head of the female. The particle displacement caused by the wing beat deflects the arista, twisting the funiculus and stimulating units of Johnston's organ at its base (Manning 1967). Manning has suggested that the aristae do not follow the frequency of the pulse (i.e. are not "tuned in"), and the important component is the frequency of pulse repetition (the time between the start of one pulse and the next). This is known as the interpulse interval

(IPI). The carrier frequency of the pulses has been shown to vary between species (Cowling and Burnet, 1981). Pulse frequency does not significantly affect female preference using muted males and simulated song playback (Bennet-Clarke and Ewing, 1969). IPI and pulse cycle are both used for species recognition and are important in species isolation (Bennet-Clarke and Ewing, 1969; van den Berg, 1988; Kyriacou and Hall, 1982, 1986; Greenacre *et al.*, 1993).

Pulse song plays a significant role in stimulating sexual receptivity in females, measured as time to copulation, in comparisons between normal males and muted males, with and without artificial simulated song playback (Bennet-Clarke and Ewing, 1967). Pulse song also reduces male locomotor activity (Von Schilcher 1976a).

One other acoustic signal is produced by both sexes of *D. simulans* and *D. melanogaster*. It is produced by males when they are the recipient of a mating attempt by other males. In females this song is seen only in virgins (Paillette *et al.*, 1991). The signal is the only reported female acoustic signal in the *D. melanogaster* complex. In both species the IPI of the signal was roughly double the length of the IPI seen in the male courtship song, and is therefore species-specific. Apart from rejection the signal plays no other role in courtship.

1.3.5 Variation in song among different species

Cowling and Burnet (1981) and Cobb *et al.* (1989) have described the courtship song of all species in the melanogaster subgroup and some of the hybrids. The plots of individual IPI of pulse song for all species, apart from *D. teissieri,* are skewed at the higher range. This is due to the spacing between pulses in each burst of the pulse song, being more uniform in the middle of the burst and longer for the beginning and end. The cause of this burst structure is unclear but may be due to the extent of the angle of wing extension, with pulses starting before the wing is fully extended (personal observation).

D. teissieri produces two wave-form elements differing from those seen in other species. The first appears to be a modulated sine song at low amplitude, but pulse song at high amplitude (IPI = 10 msecs). The second element is a pulse song composed of primary and secondary pulses, with the secondary pulses occurring 180^{o} out of phase between the first. IPI is measured between the primary pulse peaks (Cowling and Burnet, 1981).

Both sine and pulse song usually occur in courtship of *D. melanogaster, D. simulans, D. mauritiana*, and *D. orena* with no fixed order, and bursts can contain either or both song types. *D. erecta* song contains both song types, but each burst contains only one song type. Sine song is produce by both wings, but pulse song is produced by only one wing, with sine song being of higher amplitude. *D. yakuba* and *D. sechellia* produce pulse song, but do not produce any sine song (Cowling and Burnet, 1981; Cobb *et al.*, 1989).

Kyriacou and Hall (1980) showed that IPI in *D. melanogaster* song follows a simple oscillating temporal pattern. Male IPI consists of a roughly sinusoidal pattern, repeated every 50-60 seconds, with a mean amplitude of about 4 msecs. *D. simulans* IPI has a cycle of approximately 35-40 sec. *D. yakuba* shows a more complicated pulse song but has been shown to have a periodic IPI cycle of 75-80 secs (Thackeray, 1989). The species specific song cycles for both of these species have been shown to

enhance mating speed using artificial playback, when compared to playback using non-cyclic IPI (Kyriacou and Hall, 1982; Greenacre *et al.*, 1993).

The existence of these rhythms was questioned by Crossley (1988) and Ewing (1988), and subsequent debate has raged in the literature. However Crossley's 'arrhythmic' stock had been incorrectly measured, and was later found to show the rhythm on subsequent analysis of the data. The debate is reviewed by Bennet-Clark (1990) and Kyriacou *et al.* (1992). Also the existence of rhythm has subsequently been confirmed by workers in other laboratories (Noor, M. A. and Aquadro, C. F., Univ. Chicago, pers. comm.; Ringo, J. Univ. Maine, pers. comm.).

The song cycles in *D. simulans* and *D. melanogaster* were found to be controlled by the *period (per)* gene on the X-chromosome. This gene controls the circadian rhythms of eclosion and locomotor activity (Konopka and Benzer, 1971), and mutations in the gene affect behaviour (Rosato *et al.*, 1994). Some shorten, lengthen and abolish rhythm (Kyriacou and Hall, 1980). Transformation of the per^0 (arrhythmic) *D. melanogaster* males with the *D. simulans per* gene rescues circadian rhythm, but the resultant rhythm length was that of *D. simulans* (Wheeler *et al*, 1991). Female *D. melanogaster* did not, however, prefer per^0 males rescued with the *D. melanogaster per* gene over those rescued using the *D. simulans per* gene, suggesting that cycles may not play a major role in female preference for IPI (Ritchie and Kyriacou, 1994b).

The *per* mRNA has been shown to control another gene's mRNA expression cycle (*Dreg-5*). However the protein oscillation phase of *Dreg-5* is different to that of the *per* protein due to different post-transcriptional control, thus showing how one gene's expression can be controlled by *per* but have a different cycle (Vangelder and Krasnow, 1996). It may be that there are a number of steps between the diurnal *per* expression and the control of the minute long circadian rhythm of IPI involving several genes.

1.3.6 Genetic basis of the control of interpulse interval

Within the *D. melanogaster* complex backcrosses can only be achieved among *D. simulans, D. mauritiana* and *D. sechellia.* These species do not produce as extensive amounts of song as *D. melanogaster,* and until recently measuring song had been done manually, which is highly time consuming. Previous song studies have therefore limited themselves to only studying the F1 hybrids. Von Schilcher and Manning, (1975) claimed that the X chromosome played a significant role in the determination of courtship song in *D. melanogaster* and *D. simulans.* However Kawanishi and Watanabe (1981) stated that the autosomes played a significant role. Both Cowling and Burnet's (1981) and Kyriacou and Hall's (1986) behavioural studies of hybrid song confirmed the importance of autosomes in the inheritance of IPI. The conclusion was further backed up by Ritchie *et al.*'s (1994) study of variation in different laboratory strains of *D. melanogaster.* Ritchie and Kyriacou (1994a; 1996) selected for differences in IPI in *D. melanogaster,* also concluding that IPI was under mainly additive autosomal control.

1.3.7 Genetic control of IPI in other *Drosophila* species.

Other groups of *Drosophila* species possess courtship song with IPI, and the differences between species in those groups have been studied. The genetic control of IPI in other groups can be compared to the results found in the *D. melanogaster* complex to examine if the genetic control seen in the *D. melanogaster* complex is unique to that complex or similar across *Drosophila* species groups.

The *Drosophila auraria* complex has similar premating isolation to the *D. melanogaster* complex and species specific IPI. Crosses between *D. auraria* and *D. biauraria* showed that the two major autosomes acted additively in their control of IPI, with the X chromosome and the cytoplasm showing no effect (Tomaru and

Oguma, 1994a). This study used only one marker for each of the major autosomes and the X chromosome which means that study of the relative contribution of different regions of the autosomes was not possible.

The *Drosophila virilis* group consists of two phylads, *virilis* and *montana*. The species in the former group display similar song and are all allopatric as well as being closely related to each other. The species in the *montana* phylad show distinct songs, a greater genetic divergence, and live in sympatry (Hoikkala *et al.* 1982).

Hoikkala and Lumme (1984) had shown that the number of pulses in a pulse train varied between *D. virilis* and *D. lummei*. Using recessive markers they showed that genes on each of the large autosomes but not the X-chromosome affected the difference between the two species.

Hoikkala and Lumme (1987) using diallel crosses between species within the *Drosophila virilis* group, from both the *virilis* and *montana* phylads, to study differences in six song characters. In the *virilis* phylad differences between species was determined mainly by autosomal genes in each trait. Through crosses between species from each phylad they found that there had been a major change in the X-chromosome which had occurred during the separation of the two phylads, allowing variation in IPI. The result of the change had led to the evolution in the *virilis* phylad for longer denser pulse trains, while sound had evolved in different directions in species within the *montana* phylad. They concluded from their results, taken together with findings from other *Drosophila* species, that song traits were important, and had evolved to become species-specific not as a by-product of the process of speciation.

1.4 Objectives of the thesis

Chapter 2 examines the genetic control of IPI between marked *D. simulans* stock and a *D. mauritiana* stock using backcrosses. Recent developments in computer technology allow detailed study of the song of both species. The results are compared to studies of the genetic control of IPI with *D. melanogaster,* and between *D. melanogaster* and *D. simulans.*

Chapter 3 repeats the artificial selection for IPI carried out by Ritchie and Kyriacou (1996) using replicate lines. The difference between stocks selected for long and short IPI had fallen. I wished to widen this by selecting over twelve generations, then fixing the difference with the use of balancers. Four replicates of each selection regime were used to examine the relative importance of drift on change in IPI over selection.

Chapter 4 examines preference of females from the selection lines and the original unselected stock from which the lines had been founded. The covariation of preference for IPI and the trait itself was examined to ascertain the response of female preference to IPI following a change in the distribution of IPI in a population.

In Chapter 5 I examined an African strain of *D. melanogaster* which had previously been shown to demonstrate premating isolation from other strains of *D. melanogaster*. IPI of the strain was measured. Female preference of the African strain and two strains with differing distributions for IPI was measured. The role that IPI and female preference to IPI plays in premating isolation of the African strain with other strains is discussed.

Heritability and evolvability of two song traits including IPI and four morphological traits are measured in chapter 6. The measures for the song and morphological traits are compared. The values for IPI are compared to values obtained from laboratory stocks. The likely response to selective pressure for differing IPI in a natural population is discussed.

Chapter 7 discusses the relevance of all results on the importance of IPI and the role of female preference for IPI on sexual discrimination and speciation within the *D. melanogaster* complex. The possible avenues for further research are also explored.

CHAPTER 2: DIFFERENCES IN IPI BETWEEN SPECIES

2.1 Introduction

The advantage of studying differences within established species groups is that the difference between species has come about over evolutionary time as opposed to having been driven by artificial selection (Orr and Coyne, 1989). So the differences seen are due to genuine selective pressures that occur in nature, and not by artificial selective pressures whose likely role in nature is unclear and speculative.

The study of mating in the *Drosophila melanogaster* complex has usually concentrated on *D. melanogaster* itself. However the species rarely produces viable offspring with other species of the group and these are always sterile with the exception of a recently discovered *D. simulans* strain (Davis *et al.*, 1996). Hybrids between *D. simulans/ D. mauritiana/ D. sechellia* crosses all follow Haldane's rule in that they produce fertile females (homozygotic) and viable but sterile (heterozygotic) males.

Cowling and Burnet (1981) first described the songs of almost all species of the *D. melanogaster* subgroup (for *D. sechellia* and *D. orena* see Cobb *et al.*, 1989). This study was carried out without the aid of computers and hence both the number of pulses from each individual, and the number of individuals was limited. The study did however do some limited investigations into the song parameters of F1 hybrids, notably between *D. simulans* and *D. mauritiana*. They found that the mean IPI of offspring from both F1 reciprocal crosses was intermediate between the two parental values, suggesting additive autosomal effects and no disproportionate role of the X-chromosome or maternal effects. The availability of computer software, and *D. simulans* phenotypic markers now allows a more in-depth study of the genetic control of the difference in IPI between *D. simulans* and *D. mauritiana*.

The female F1 hybrids are viable and fertile and can therefore be used in backcross experiments. The F1 adult males, while sterile, are viable, and will still

court females freely, producing courtship song. The simulans clade also makes an interesting group to study as much work has been done on pheromones, genital arch morphology and in postmating systems, notably sperm viability (e.g. Liu, 1989; Zeng and Singh, 1993a), while little has been done on song differences and their preferences between species.

D. sechellia have poor viability on the medium used in the laboratory, and although it has been found to produce song, it sings much less readily than *D. mauritiana*. I was unable to record any song from *D. sechellia* so therefore used *D. mauritiana*. *D. mauritiana* has about half the average pulse number of *D. simulans*, but it is enough to make reliable IPI measurements per individual. As the F1 females were being backcrossed to the *D. simulans* parents it was hoped that the higher rate of singing in this species would also be reflected to some degree in the offspring.

The genome of *D. simulans* and *D. mauritiana* consists of four pairs of chromosomes. The fourth (dot) chromosome is extremely small containing only 0.07% of the genome, and has been ignored for this study. The X-chromosome consists of over twenty percent of the genetic information, with the second chromosome being slightly smaller than the third chromosome, with both being just under twice the size of the X-chromosome. (Ashburner, 1989). The probable chromosome base number is five equally sized chromosomes, with two pairs having fused (not including the dot chromosome).

By using a strain of *D. simulans* which contained recessive phenotypic markers on each arm of the two major autosomes and one on the X-chromosome it is possible to split up the genome into five roughly equal areas to which differences could be mapped. While there was a possibility of recombination producing areas within the marker region that might not contain *D. simulans* parental DNA this effect was ignored in this study, this has also been the case in other studies (e.g. Orr, 1987). Recombination also allows the markers from each arm of the major autosomes to disassociate and the individual effects of each arm of an autosome could be examined independently.

If the IPI is controlled by several genes distributed evenly through the genome, then it would be expected that the X-chromosome, and each arm of each autosome, would contribute to roughly a fifth of the difference in IPI between the F1 and *D. simulans* values.

Many studies have been made of pheromone blend differences between *D. simulans* and *D. sechellia*. Here the case has been very much one of major region effect. Although pheromones and courtship song are both involved in sexual isolation between species, male production of each pheromone type, and their relative ratios both seem to be under the control of a few regions of major effect, while reception of particular pheromones in females is controlled by the number of receptors. Courtship song is produced by physical means i.e. flight muscles and associated neurological control. The reception is of a physical disruption of air particles, again triggering a neurological pathway. Also the use of the flight muscles and wings involves systems that are under other selection pressures besides sexual selection including many that have direct relevance to general fitness, although cuticular hydrocarbons are also important in protection from desiccation.

One factor of the mating song, the period of cycles of mean IPI, has however been shown to involve a single gene of large effect. The *period (per)* gene has been shown to be sex linked and responsible for the difference of cycle length between species (Wheeler *et al.*, 1991), and females prefer the period of conspecifics (Kyriacou and Hall, 1986; Greenacre *et al.*, 1993).

A study of the difference in mean IPI between *D. auraria* and *D. biauraria* of the *D. auraria* complex, found control was additive and autosomal; however, this used only three markers (one per chromosome) and is therefore of limited resolution (Tomaru and Oguma, 1994a). This species complex also has a species-specific IPI and it IPI has been shown to be important to female preference (Tomaru and Oguma, 1994b).

2.2 Materials and methods

2.2.1 Stocks

The *D. mauritiana* stock was obtained from Prof. J. David, Gif, France (David *et al.*, 1989). The wild type *D. simulans* fly stocks used for the crosses were Seychelles obtained from Prof. J. David, and Leticia (Columbia) and Ruratonga, (Cook Islands), obtained from Dr. C. P. Kyriacou, Leicester University. The mutant strain was the multichromosomal mutant stock S41 (f^2;*nt, pm; st, e*), obtained from the *Drosophila* species stock centre, Bowling Green, Ohio. All stocks had been in laboratory culture for several years

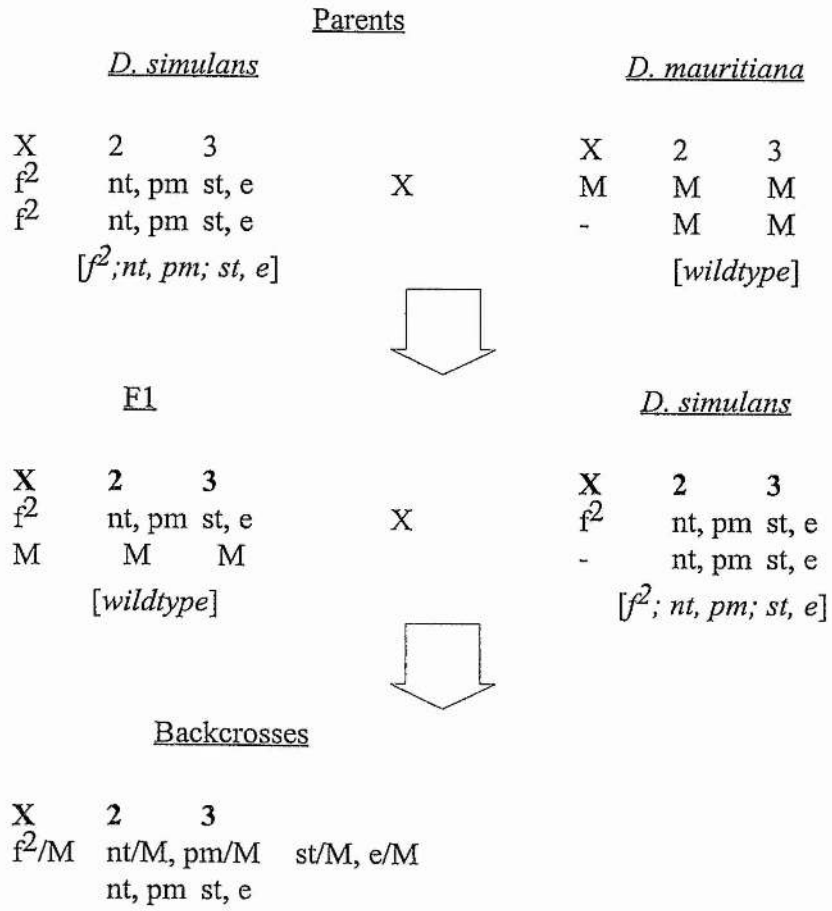
The mutant strain contained recessive mutant markers, which were as follows; f^2(*forked*, twisted, short bristles) on the X chromosome (Map reference: 1-56.7); *nt* (*net*, wing venation) left arm of the second chromosome (2-0.0), and *pm* (*plum*, eye colour) on the right arm (2-104.5); *e* (*ebony*, body colour) on the right arm of the third chromosome (3-70.7) and *st* (*scarlet*, eye colour) on the left arm (3-44). When *net* and *plum* phenotypes appear in the same individual the eye colour appears creamy orange. The fourth dot chromosome was not marked, and only contains less than 0.07% of the genome. All mutations used did not involve large changes in wing or muscle morphology that might affect the mating song.

All stocks were each maintained in two replicate third pint milk bottles containing the standard yeast sugar *Drosophila* medium (10g agar, 65g sucrose, 15g dried active yeast, and 10 mls 30% Nipagen in 1l water) on a 12/12 hour light/dark cycle at 25^{o}C. Further generations were established by mass transfer of between thirty to forty individual adults to new bottles containing fresh media, although exact numbers were not recorded.

F1 stocks were established by mass mating ten virgin *D. mauritiana* males with ten virgin *D. simulans* homozygous multiple mutant females from the S41 stock.

Backcross stocks were set up using ten virgin F1 females (which were from the same bottles as the F1 males recorded) with ten virgin *D. simulans* homozygous multiple mutant males per cross. The backcross offspring males were examined to determine which markers they possessed and their song was then recorded and analysed.

The backcross protocol is shown in figure 2.1 below.



M = *D. mauritiana* chromosome

[*phenotype*]

Figure 2.1 Genotypes and phenotypes of the backcross protocol. Backcross phenotypes are shown in table 2.3.

2.2.2 Song analysis

Virgin males were isolated on eclosion into glass vials containing standard sugar/yeast medium , and after at least two days were then placed in the microphone chamber of an insectavox for at least one minute in order for their body temperature to equilibrate with the ambient temperature of the insectavox.

The insectavox consists of a microphone chamber (13 cm x 13 cm x 10 cm) that is lined by foam to insulate the chamber from external noises, and has a latched lid for access containing a convex observation window to allow direct observation of the mating pair during courtship. The microphone is located in the middle of the chamber on a raised dais, and records the particle displacement of the air. The light source is located in an area next to the chamber and the light passes along the length of a solid perspex cylinder to the dais in order to provide a cold light source. The thermocouple from an electronic thermometer is positioned next to the microphone on the dais and measures the ambient air temperature of the chamber (accurate to $\pm 0.2^{o}C$). The flat cylindrical courtship chamber (8 mm diameter x 3 mm) is made of perspex with a transparent gauze floor and a cotton wool stoppered entrance. This is placed on the dais directly over the microphone, illuminated by the light source. With the exception of the electronic thermometer, the general set-up of the insectavox is that described by Gorczyca and Hall (1987).

The male was then placed into the courtship chamber, without anaesthetic using a pooter, with a virgin female from the *D. simulans* parental line which had been collected within 24 hours of eclosion under CO_2 anaesthetise. The females were muted upon collection by amputation of the wings using a scalpel just above the point of attachment to prevent any damage to the muscle in the thorax. Once the male had produced one burst of pulse song, the next five minutes of courtship was recorded. Variation in IPI cycles with a period of about 30 to 40 seconds in *D.* simulans (Kyriacou and Hall, 1986) and the sampling of five minutes of song insures that all

parts of the cycle are sampled. All recordings were done under constant light as visual cues are also important in courtship.

The songs were recorded using cassette tape in a Marantz recorder with a set recording level, then played into a PC compatible computer after the analogue signal had been filtered using a high pass of 250 Hz, and a low pass of 1 KHz. The lower level was set in order to filter out much of the background noise which is usually of lower frequency, and also much of the sine song. The higher range was set at half of the sampling rate of digitization to prevent aliasing of high frequency components.

The signal was digitalized by a 1401 analogue-digital converter, using a sample rate of 4 MHz, and then stored as a SPIKE2 formatted file into a PC-compatible computer. The temperature was noted using the thermometer inside the microphone chamber at the start and end of every recording, with the temperature taken to be the average of both. Variation between start and finish temperature was rarely greater than the range of accuracy of the thermometer.

All wild caught stocks were recorded within each recording session to avoid differences due to environmental effects in any single recording block resulting in bias in the results. Individuals from each stock were interleaved with those of other stocks when recording so individuals of each stock were represented evenly in each block of recordings. The same tape recorder was used thoughout recording and digitization.

The files were analysed using SPIKE2 (C.E.D.) software, using a prewritten analysis program FASTDMEL.TXT. This is a semi-automated program which allows the user to view the song waveform. Events channels are created which store the onset of events using amplitude threshold crossing criteria. The threshold was set manually above the amplitude of the background noise, but low enough to be triggered by higher amplitude pulses. There was then a 0.02 msec gap when no further events were recorded. This prevented subsequent false events being recorded which were due to oscillations of the waveform from the same pulse.

The analysis program then allowed the operator to view a histogram showing the distribution of the pulses. The upper and lower cut-off points defining the position

of the maximum and minimum IPI values to be subsequently analysed were manually set around the upper and lower ends of the distribution curve to give a reasonably normal IPI distribution. Although the positioning of these limits greatly affects the resultant mean IPI value, this is necessary due to the distribution of the histogram which is 'right sided' and cannot be corrected by transformation (see Cowling and Burnet, 1981). Repeated assigning of limits to any one distribution give highly similar results The computer then computed the mean value (IPI) for all event intervals occurring between these cut-offs. A printout of this data was obtained showing the histogram of the IPI distribution, the IPI value, temperature, number of IPIs and phenotypic marker (for backcrosses only).

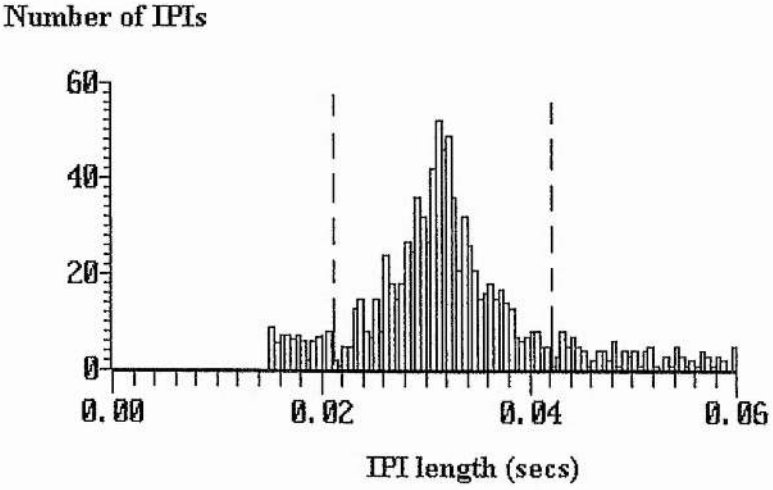


Figure 2.2 The distribution of IPI lengths from an individual recording of courtship song. Dotted line represent upper and lower cut-off points for measurement of upper and lower range of the distribution curve.

If there were too many spurious events due to background noise the global events line could be reset. If this did not improve the ability to distinguish the distribution curve, the recording could be analysed using EVENT.TXT. This allowed

the operator to examine the song trace and to remove events (due to loud background noises), and add events (due to pulses of low amplitude not reaching the event line amplitude) to global event line. Once completed the histogram was again examined and if satisfactory, a printout obtained.

The distribution of IPIs is skewed towards the longer values, especially in *D. mauritiana*, with longer IPI's observed at the beginning and end of the pulse bursts. Bursts follow the pattern of longer IPI's initially, shortening rapidly, then followed by a core of evenly spaced pulses with shorter IPI's before lengthening again at the tail-end of the burst. It has been speculated that the modal and not the mean IPI is the parameter that is used by females (Cowling and Burnet, 1981).

The resulting data were used to determine the group mean IPI using MINITAB, and the mean and modal IPI for each strain compared. The values of the IPI were regressed on temperature and were not adjusted for temperature if the values were not significant.

All SPIKE2 analysis programs were written for use in *D. melanogaster* song analysis (Ritchie and Kyriacou, 1996) and have been checked for accuracy (Ritchie and Kyriacou, 1994).

2.3 Results

The temperature range for wild type strains was 22.5 - 27.5°C (mean = 25.54°C, S.D. = 1.2°C), and for the mutant strain was 24 - 27.5°C (mean = 25.61°C, S.D. = 0.83°C). There was no significant correlation of modal IPI with temperature, and no correction was made, this may be due to the low range covered.

The mean and modal IPI of each individual recording was compared for all individuals from the wild strains of *D. simulans* (figure 2.3) and was found to show a significant regression ($r = 0.94$, $F_{1,67}=37.84$, $P<0.001$). As there was a significant regression the modal IPI value was used as the standard measurement for the IPI value of each recording. Each individual's modal IPI value was treated as a parametric data point, and subsequent analysis of population IPI values was by parametrical analysis.

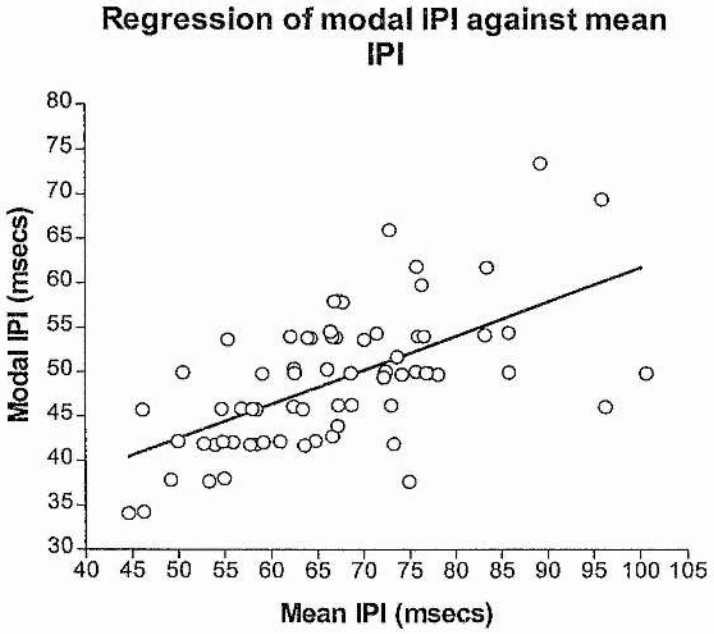


Figure 2.3 Regression of mean and modal IPI of all individuals from wild derived stocks of *D. simulans*.

The IPI of the mutant stock was within the range of wild caught strains (Table 2.1). One way ANOVA shows that although there was significant variation between the four stocks ($F_{3,62} = 4.56$, $P<0.01$), the difference between the pooled data (which has been used as the effective species IPI value) and the mutant S41 strain was not significant ($t= 1.16$, d.f. = 59, NS), and therefore it was assumed that the mutations possessed by S41 males did not affect IPI and that the mutant stock could therefore be used as a representative strain of *D. simulans* in species crosses.

D. simulans stock	N	IPI	S.E.	Range
Seychelles	15	52.38	2.50	37.69-73.45
Leticia	16	54.53	1.46	46.07-69.46
Ruratonga	14	46.50	1.14	37.69-51.71
POOLED	45	51.31	1.14	37.69-73.45
Mutant	21	49.55	1.02	41.85-58.02

Table 2.1 shows the overall IPI for the individuals measured. Pooled contains data from all wild type strain individuals

The temperature range of the *D. mauritiana* recordings was 24 - 27°C (mean = 25.4°C, S.D. = 1°C). There was no significant correlation of IPI with temperature, therefore IPI was not corrected for temperature.

The temperature range for the F1 hybrid recordings was 23-27.75°C (mean = 25.05°C, S.D. = 1.12°C). The eighteen F1 male recorded were all from *D. simulans* females x *D. mauritiana* female crosses, as the reciprocal cross produced very few males, none of whom sang (as seen by Lemeunier *et al.*, 1986).

There was a significant difference ($F_{2,51} = 44.37$, $P<0.001$) between the IPI of the parental and F1 stocks using one way ANOVA (Table 2.2). The F1 value did not differ significantly from the midparent value ($t = 0.46$, d.f. = 17, NS). This result agrees with that found for modal IPI between the two species and the F1 offspring found by Cowling and Burnet (1981).

Species	*N*	IPI	S.E.	Range
D. mauritiana	15	35.7	1.04	29.86-42.19
D. sim (s41)	21	49.55	1.02	41.85-58.02
F1 hybrids	18	41.85	0.28	34.08-46.33

Table 2.2 shows the overall IPI for the parental stocks and F1 offspring measured.

All backcross individuals were grouped together according to the markers possessed, and covered 26 of the possible 32 groups. The modal IPI of the different gene marker groups with the same number of markers were then analysed to determine if there was a significant difference between the groups (table 2.3). Using one way ANOVA no significant difference was found in any of the groups containing the same number of markers; ($F_{3,37} = 0.88$, NS) for one marker; ($F_{6,37} = 1.02$, NS) for two markers; ($F_{4,24} = 1.29$, NS) for three markers; and ($F_{1,6} = 1.58$, NS) for four markers. This does not suggest any epistasis. However a significant difference was found ($F_{5,161} = 12.62$, $p<0.001$) between these groups.

Marker	Number of markers	Modal IPI	± S.E.	P value
Wild type	0	39.50	1.48	-
st	1	42.60	1.66	0.462 (N.S.)
nt		43.17	2.71	
pm		44.39	1.5	
f		41.18	1.3	
st, e	2	46.89	2.65	0.536 (N.S.)
nt, e		53.00	3.79	
st, pm		46.00	-	
pm, nt		46.56	1.4	
f, e		46.00	4	
f, st		43.25	3.9	
f, nt		49.00	3.42	
f, pm		44.83	1.88	
st, e, nt	3	46.50	3.1	0.342 (N.S.)
st, e, pm		49.80	1.8	
e, pm, nt		42.00	-	
st, pm, nt		46.00	-	
f, st, e		52.00	2.03	
f, e, nt		46.00	-	
f, st, nt		42.00	-	
f, st, pm		50.00	4	
f, pm, nt		47.40	1.29	
f, st, e, nt		44.00	-	0.272 (N.S.)
f, st, e, pm		49.50	2.87	
st, e, pm, nt	4	54.75	3.04	
f, st, e, pm, nt	5	56.80	5.19	-

Table 2.3 The IPI of males grouped by their marker phenotype. The P value is from a one way ANOVA between groups with equal number of markers

T Tests were then carried out on each group to see if they varied from the expected value, shown in table 2.4 below, being the value expected from equal contribution of each marked area to the difference between the wildtype (no markers) phenotype and the *D. simulans* f, st, e, pm, nt phenotype.

No significant difference was found (see table 2.4 below), therefore each marked area of the genome can be assumed to contribute equally to the IPI, suggesting a polysomal additive effect of genes located throughout the genome.

No. of markers	N	IPI	S.E.	Range	Expected value	p value
0	20	39.50	1.48	30 - 54	39.50	-
1	41	42.66	0.84	30 - 54	42.20	0.54
2	45	46.62	0.98	32 - 62	45.94	0.06
3	33	48.52	0.89	38 - 61	47.66	0.39
4	9	51.22	2.12	44 - 60	50.38	0.41
5	5	56.80	5.19	42 - 70	56.80	-

Table 2.4 The IPI of flies grouped by marker number

Figure 2.4 Illustrates the stepwise increase in IPI with the increase in number of markers, and hence the proportion of *D. simulans* chromosome possessed. This is indicative of the expected result from an additive autosomal model with no epistasis, with figure 2.5 showing the IPI of males grouped by marker identity and both the groups with (clear bars) and without (hatched bars) the X-linked marker reflecting the general trend of the IPI to increase with marker number, and no disproportional effect of the X chromosome.

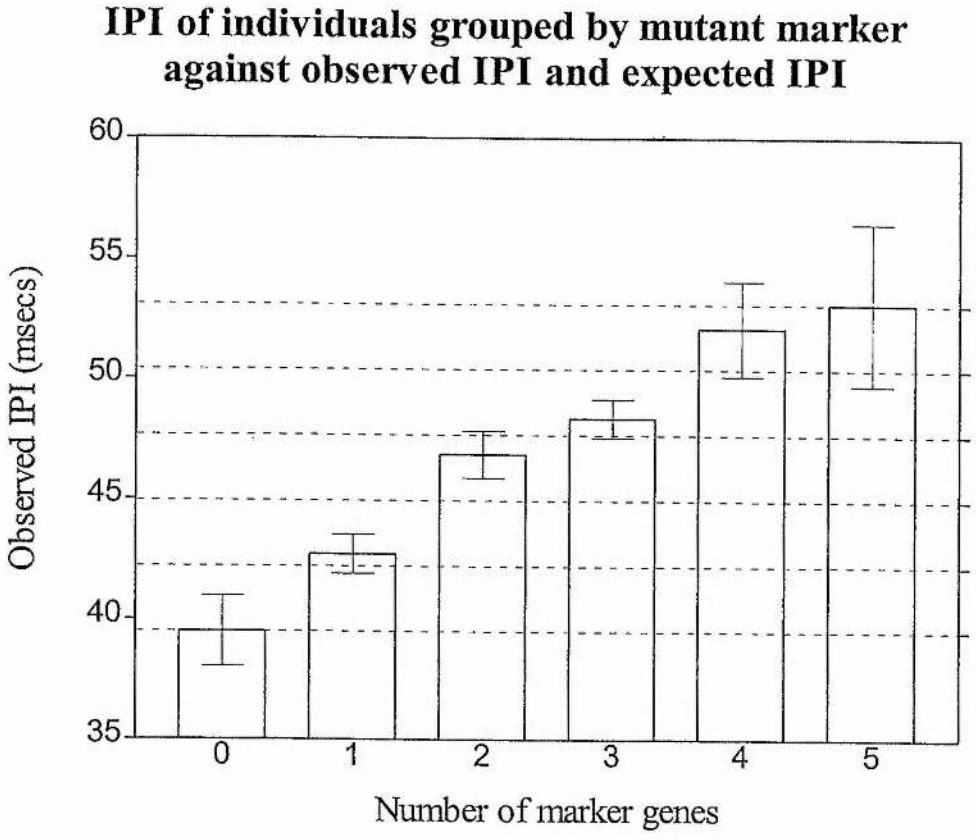


Figure 2.4 IPI grouped according to number of phenotypic markers possessed and expected IPI value, assuming equal affect by each marked region (dotted lines).

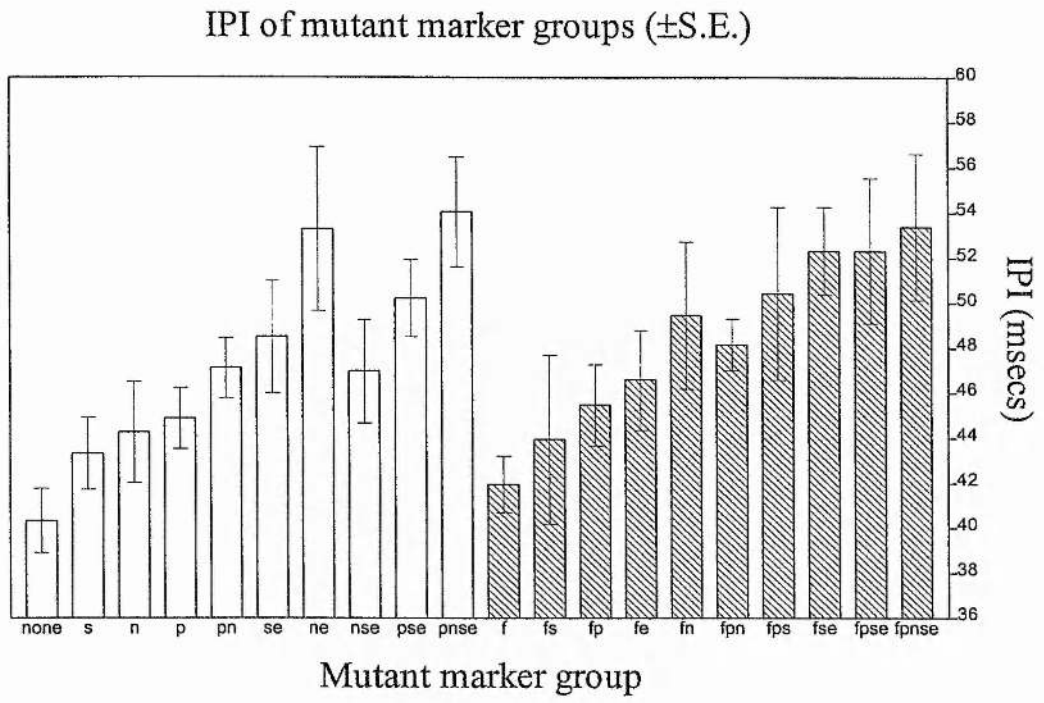


Figure 2.5 IPI of individuals grouped by markers possessed. Groups with less than two individuals were not included. Plain columns do not possess the forked (f) marker and therefore possess the *D. mauritiana* X-chromosome, and hatched columns possess the forked (f) marker and the *D. simulans* X-chromosome.

2.4 Discussion

The results from the comparisons within and between backcross males grouped by marked number suggests that the difference in modal IPI between the songs of the two species is controlled by additive genes distributed evenly throughout the genome, in the X-chromosome and the two major autosomes. No evidence was found for a disproportionate contribution by the X-linked marker (i.e. the X-chromosome), or epistasis between the different genomic regions.

For this study the effect of each region is described as being equal if each marker area played a role in the difference in IPI that was not disproportionately large in comparison to the relative proportion of genome of that area. The effect of a region being described as major is defined as that which constitutes a significantly larger effect in comparison to the area's genomic proportion.

It is impossible to resolve the number of genes that contribute to the difference of any region from this experiment, and therefore the confidence level is *no less than* five genes or gene groups in the entire genome contributing to the difference. There may be as few as five genes responsible, one in each region, which would still constitute genes of major effect. From the results of this study, it is impossible to know the number of genes contributing to the overall difference of each marker area.

The study of IPI in the *D. auraria* complex which used a similar method but with only one marker for each of the three chromosomes found a similar result (Tomaru and Oguma, 1994a). This also seems to be the case for assortative mating between recombinant genotypes of *D. sechellia* and *D. simulans* (Coyne, 1992a).

This contrasts with the studies of 7-tricosene/7-pentacosene blend difference between *D. simulans* and *D. sechellia* which have suggested a role for major genes on the third chromosome and also for female preference for the Tai-Y and CS male pheromonal phenotype of *D. melanogaster* being influenced by a gene, or genes, on the third chromosome (Coyne *et al*, 1994; Scott, 1994). Single genes of large effect have also been seen in moth pheromonal studies (Löfstedt *et al*, 1989; Löfstedt, 1993).

It may be that the relative expression of different compounds in such chemical communication systems are much more likely to be controlled by relatively few genes than those that control length of IPI in song.

Shaw's (1996) recent study of Hawaiian cricket species found that the pulse rate of the song of males in crosses between *Laupala kohalensis* and *L. paranigra* differed significantly in reciprocal crosses, suggesting an X-chromosome contribution (an average of about 11%, roughly proportional to it's size), and she estimated roughly eight genetic factors were responsible for pulse rate differences between the two species.

There may not be a general pattern for sexual isolation, and any pattern will be limited by the mode of signal transmission and reception, with different rules for pheromonal and acoustic communication systems.

The 'genetic coupling' hypothesis (Alexander, 1962) suggests that genes responsible for controlling a trait, and the preference for that trait will become closely linked, with both even being controlled by the same gene. The polygenic nature of a signal would mean that the 'genetic coupling' hypothesis would be unlikely to be the main cause of shift in signal and preference between the two species. Even in systems with major genes there may still be no linkage as in pheromone production and reception of the European Corn Borer (Löfstedt *et al.*, 1989).

Bradshaw *et al* (1995) demonstrated that major genes affect reproductive isolation between two monkeyflower species *Mimulus cardinalis* and *M. lewisii*. The two species are fertilized by hummingbirds and bees respectively, and Bradshaw *et al.* see a plausible explanation being that genes of major effect allowed the large changes in the flower needed to change the morphology and nectar production to move from being attractive to insect pollinators to being attractive to bird pollinators. This can be explained using Wright's adaptive landscape model, although mutations of large effect are usually considered by the model to be more likely to be deleterious, as they move the phenotype further from the adaptive peak, in this case they might have allowed change large enough to move the flower phenotype on past the maladaptive trough

and across to the slope of the bird pollinated adaptive peak. Later small mutations would have then 'fine tuned' the flower phenotype to reach the higher regions of the new adaptive peak. The change is analogous to that of a cryptic and mimic model with it's two adaptive peaks. This would seem to be much less likely for the differences in IPI of the two species studied here, as there are no clear external selective forces that would result in such a clear adaptive bimodal topography as that seen in the monkeyflower.

QTL analysis will be important in increasing the resolution and resolving the contribution of different parts from one region, and eventually locating the individual genes that control the traits. Also the ability of QTL analysis to separate the genome into smaller regions will allow a more meaningful study of potential linkage of genes involve in the preference for a trait, and those responsible for the control of the trait. The use of QTL markers in evolutionary studies over the last few years have shown that some traits which were thought to be polygenic have been shown to involve major genes (Bradshaw *et al*, 1995; Mitchell-Olds, 1995; Liu *et al*, 1996). Liu *et al.* (1996) suggest that the use of a more finely tuned technique, such as QTL analysis, will show signs of higher complexity of the genetic control of regions studied. The results will show dominance and epistasis effects that are unable to be resolved by the previous techniques.

Liu *et al* (1996) looked at differences in genital arch morphology, thought to be important in controlling the length of copulation and hence amount of sperm transferred, between the same two species as this study, using eighteen marker loci and found that eight of the fifteen intervals showed evidence of QTL's with an effect of between 5.7 - 15.9%. They found that this was largely additive, but with some dominance of *D. mauritiana* alleles and epistasis. The relatively large effect of these areas was not shown in previous studies due to their reduced resolution..

It would seem likely that many causes of sexual isolation, like differences in IPI, are polygenically determined, and would have evolved relatively slowly, most likely in allopatry as suggested by Orr and Coyne (1992). The differences in IPI

length between *D. mauritiana* and *D. simulans* (and most likely *D. sechellia* and *D. simulans*), would have evolved following the establishment of a founder population on the islands from the African continent.

The song of *D. mauritiana* has a shorter IPI compared to *D. simulans*, and *D. melanogaster* females may prefer shorter song because it might be used as a fitness measure. I have described in the introduction how Ryan and Rand (1993) pointed out that females could prefer a signal outwith the present range of the signal produced by conspecific males, e.g. as with a 'super-normal' signal. The ancestors of *D. mauritiana* may have had a signal similar to that of *D. simulans,* and females may have been selecting for shorter IPI length, with other factors exerting counter selection with this trade-off defining the lower limit of the signal range. The bottleneck of the founder event, and the removal of some environmental or genetic constraint would have then allowed males with shorter song to be selected for. Without the antagonistic selection pressure the female preference distribution would have shifted to a lower range than that seen in the ancestral population, and towards that now seen in *D. mauritiana.*

One possible constraint could have been the selection against song within the range of *D. melanogaster* with which *D. simulans* is sympatric, and whose upper end of the range slightly overlaps with the lower end of the range of *D. simulans. D. melanogaster* has until recently been absent from the Mauritius islands, and it has been noted that insemination of *D. melanogaster* females by *D. mauritiana* males was seen where populations occur sympatrically (David, 1981). It may be that the loss of sexual isolation due to the displacement of female preference for a higher IPI in the ancestral populations of *D. mauritiana* is the reason that wild hybrids now occur. It would be interesting to see what the female preferences of *D. simulans* was over a range beyond it's own species signal range and if there was any preference for shorter song.

Future work between the two species should benefit from molecular studies, but the number of markers is limited, especially in *D. mauritiana*. The number of markers that are used in molecular studies, such as DNA repeat sequences and

RAPD's may be still be limited due to the likelihood of great similarity between the species due to their recent divergence, and hence a large degree of homology in sequence in both species. The study of *D. melanogaster* crosses with *D. simulans* (and perhaps the other two species in the Simulans clade) may now be possible (see Davis *et al.*, 1996). Also the song of *D. melanogaster* contains many more pulses per minute compared to that of *D. simulans* and especially *D. mauritiana,* making analysis easier, without the need to manually check the results from automated analysis.

CHAPTER 3: ARTIFICIAL SELECTION FOR IPI IN *D. MELANOGASTER*

3.1 Introduction

Several studies of artificial selection in *D. melanogaster* have examined traits involved in mating speed, i.e. sexual activity. For example, two traits which have been studied are male percentage wing vibration (which produces courtship song) and licking (the occurrence of which is closely associated with song production). The amount of sexual activity is usually linked with general male vigour (Manning, 1961). It may be that females use sexual activity as a cue to the general fitness of males. This is likely to have resulted in high directional selection by females for increased sexual activity.

Fisher's Fundamental Theorem of Natural selection (Fisher, 1930) states that 'the rate of increase in fitness of any organism at any time is equal to its genetic variation in fitness at the time'. Therefore populations under continued selection would be expected to exhibit low additive genetic variation in fitness. This follows from Kimura's generalisation of the theorem (Kimura, 1958), and genetic variation is therefore due to dominance or epistasis (Charlesworth, 1987). Measures of total fitness are practicably impossible. Partial measures of fitness are therefore used. However partial measures of fitness are perceived as being potentially problematic because different components are often negatively correlated (Rose 1984; Partridge and Fowler 1992, 1993). However it has been seen that high levels of additive genetic variance have been maintained even in the presence of strong sexual selection due to female choice (Mousseau and Roff, 1987). Pomiankowski and Møller (1995) suggested that these high levels could be maintained if the directional selection is greater than linear, as this selects for genes which increase phenotypic variability. Additive variance (V_A) is expressed in terms of heritability (h^2) scaled by total phenotypic variance (V_P) [$V_A = h^2 * V_P$]. A low heritability may therefore only be reflecting a high degree of residual variance that masks the level of additive variance.

It has therefore been suggested that a more appropriate measure of 'evolvability' would be the coefficient of additive genetic variance (CV_A) which standardizes the variance with respect to the mean [$CV_A=100\sqrt{(V_A/mean)}$] (Houle, 1992). Significant additive variance is seen for fitness components (Falconer and MacKay, 1996), but shows lower heritabilities on average than other characters (Mousseau and Roff, 1987; Falconer and MacKay, 1996).

Artificial selection for sexual activity levels is usually more successful when it is for reduced activity, and Frankham's (1990) review of bi-directional selection for reproductive fitness characters found that 24 out of 30 experiments had resulted in asymmetrical responses to selection, with a greater response to selection for lower fitness traits. He argued that the asymmetry was most likely due to strong directional selection, not only by female preference but also by other environmental factors selecting for general fitness. Selection against genes that reduce activity would mean that only recessive deleterious genes would remain in the population as they would have been partially shielded. Dominant genes that reduce activity would have been strongly discriminated against within natural populations. Even recessive genes of major effect, would only be discriminated against when homozygous and Frankham (1990) suggests that the rapid response seen in the lines selected for lower fitness could be due to recessive deleterious genes of major effect, which are likely to be rare, but able to produce the large responses seen. The use of realized heritabilities calculated from the divergence of lines during bi-directional selection removes asymmetry due to differences in the response to directional selection in one direction only against a control line. However artificial selection on any trait which is associated with general fitness is complicated by the fact that fitness in all lines decreases through the course of selection due to inbreeding, thus enhancing the appearance of asymmetry with greater response for less fit individuals.

McDonald (1979) selected for bi-directional change in percentage wing vibration (PWV) of *D. melanogaster* for eleven generations using two replicate lines per selection regime, and found that both replicates for decreasing PWV produced

reductions, but an increase was only seen in one of the replicate lines selecting for increasing PWV. Realized heritability for the divergence between the selection regimes was 15% for the first 11 generations. There were also signs of inbreeding depression. McDonald and Crossley (1982) continued the selection for a total of 44 generations, and found that changes in PWV were due to changes in bout (burst) length. Moreover, the change in PWV was associated with change in licking rate, suggesting that these two components are possibly linked.

One interesting point is that one of the high PWV replicate lines showed a significantly longer interpulse interval (IPI) than the corresponding low PWV replicate line, 41.4 msecs compared to 39.1 msecs. However the control line was not measured, and there was large temperature variation. Both these values would seem rather long for the species, but this is due to the recording temperature (21^{o}C) (the species IPI is usually given as the value seen at 25^{o}C). Adjustment of the values for temperature gives IPIs of 32.1 msecs and 34.4 msecs respectively, both of which are within the normal range seen in *D. melanogaster*. It may be that the difference in IPI is due to drift or indirect selection for longer IPI with higher PWV, especially as it was not seen in the other replicate line. It may be that the high energy cost of increasing PWV has been obtained to some extent by reducing the pulse rate within bursts, and hence producing the longer burst length for the same number of wing beats and energy input.

Licking of the female's genitalia by males increases during or after wing vibration and usually precedes mating attempts (Cobb *et al.*, 1986?; Welbergen *et al*, 1987). Licking is regarded as an indicator of sexual activity, because low licking rates are correlated with slower mating rates (Manning, 1961). Welbergen and Van Dijken (1992) selected for high and low licking rate of courting males of *Drosophila melanogaster* for 21 generations. Asymmetry of response to the two selection regimes was seen with the low licking rate line initially responding linearly to selection before reaching a plateau after eight generations, with no significant change being seen in the high licking rate line. The realized heritability of the low and high licking rate line

over the first seven generations was 41% and 0.4% respectively. The licking rates of the lines differed significantly from generation four onwards. X-chromosome, maternal effects, and dominance were not found to be involved. High line males mated significantly faster with standard females than did Low line males.

Ritchie and Kyriacou (1994) studied mean IPI of the courtship song of *D. melanogaster*. Twenty seven generations after collection, a father-son regression showed covariation of IPI was 5.4%, with a low heritability estimate of -0.15 (SE=0.308), being non-significant. Twenty isofemale lines had been established, and after thirty generations significant variation was seen between lines, although there was no increase in the total level of variability across all lines. Four males from each isofemale line were recorded and males from all lines were ranked by mean IPI. The four highest and four lowest ranked males from all lines were mated to a single female to create the long and short lines respectively, and ten offspring males from each of the lines were recorded. Response to selection was significant showing that some of the variation seen between the isofemale lines was genetic and additive. Estimates for realized heritability gave values of 43% and 47% for the short and long lines respectively.

They continued selection for a total of six generations, resulting in a significant difference in mean IPI between the two lines (Ritchie and Kyriacou, 1996). Response to selection was asymmetrical, with the long line showing the greater response. Analysis showed only significant additive autosomal effects and a smaller X-chromosome effect (3.3ms and 1.3ms respectively). Divergence decreased over the course of selection, possibly due to limited genetic variation. The short and long lines were approaching non-overlapping distributions by the last generation of selection, and for the long line, the mean was well beyond the recorded range for wild strains of *D. melanogaster*. F2 and backcross generations showed continuous distributions with no indication of overdominance or linkage effects. Analysis of response to selection gave a realized heritability of 26.2% with a sampling variance of 0.3%. Phenotypic variance was 2.14, implying an evolvability of 2.1%

There was a decrease in number of pulses in the long line larger than expected for the decrease in IPI. This meant that as the burst length was unchanged there were fewer pulses per burst, i.e. the PWV is unchanged. However replicate lines were not used so it is unclear how much of the difference between the two lines is due to genetic drift.

The aim of this experiment was to repeat this selection with replicate lines to examine if response to selection was correlated across replicate lines showing a real response to artificial selection, thus ruling out change being due to genetic drift. Drift could not be estimated in the experiment by Ritchie and Kyriacou (1996). Replicate lines also allow examination into the covariation of female preference of selected lines to males displaying different IPI's.

Since the first artificial selection experiment, the stocks had been relocated to St. Andrews University from Leicester University, and no work had been carried out on the selected lines for two years. The difference between the two lines had fallen from the end of artificial selection by more than 3 msecs to just over 1 msec. Renewed selection should result in an increase in the difference which would facilitate studies into the genetic nature of the difference between the lines. Once the difference had been enlarged this difference would be fixed by chromosome isolation to prevent subsequent convergence in IPI. Although the importance of replicate lines is emphasised during the course of selection, replicates were not used for chromosome isolation, as the aim was to fix differences that had already been quantified.

3.2 Methods

3.2.1 Stock Maintenance.

D. melanogaster from Pietrastornina, Italy was collected in 1989. Lines were established with mass transfer and thorough mixing in subsequent generations. This stock is hereafter referred to as the ancestral "Out" stock. An initial round of artificial selection over six generations for short and long IPI over six generations produced the two lines selected for short and long mean IPI, called "Short" and "Long" with a difference of over 4 msecs in mean IPI between selected lines (Ritchie and Kyriacou, 1996). After the stocks had been moved to St. Andrews University the Long, Short, and Out stocks were each maintained by mass transfer in two replicate milk bottles (third pint) containing the standard yeast sugar *Drosophila* medium for twenty seven generations with no artificial selection pressure. Development time was not regulated, but did not vary significantly between stocks. All stocks were kept at 12/12 hour light/dark conditions, and at 25^{o}C thoughout the entire experiment.

3.2.2. Song Analysis

Recordings of the courtship song of males from twenty seven and twenty eight generations after the start of the initial selection experiment were made in order to measure the mean IPI of the Long, Short and Out lines since the end of the selection experiment. After a further 26 generations (generation 53 after selection started) the courtship songs of forty males each from the Long and Short lines were recorded, and the mean IPI of the lines calculated.

Males from the three lines were anaesthetised by CO_2 and isolated within twenty four hours of eclosion in 30ml glass vials containing medium, and after two days the vials were placed in the microphone chamber of the insectavox for at least

one minute in order for their body temperature to equilibrate with the ambient temperature of the insectavox. A single male was then aspirated into the courtship chamber without anaesthetic, which already contained a virgin female from the Out line. The female had been collected within 24 hours of eclosion and made mute by amputation of the wings under CO_2. All females were used within twenty four hours of collection. Recording was started once the male had produced one burst of pulse song, and continued for the next five minutes provided that the male was actively courting for most of this period. The temperature was noted using a thermometer inside the microphone chamber at the start and end of every recording, with the temperature taken to be the average of both. The temperature did not vary beyond $\pm$ 0.2°C. Temperature was held close to 25°C by the use of fans and heaters. For full details of the recording set-up and the microphone (insectavox) see chapter 2 and Gorczyca and Hall (1987). All recordings were done under constant light and during the twelve hour light period with all lines recorded in every session and males from each stock sequentially interleaved. Although the results were not tested for variation with time of day, as all lines were represented in all sessions it was presumed any variation would not contribute to variation between lines.

The songs were 'recorded' straight into an IBM-compatible computer as a SPIKE2 formatted file after the analogue signal had been filtered using a high pass of 250 Hz, and a low pass of 1 KHz, and digitized by a 1401 analogue-digital converter using a sample rate of 4 MHz. The files were then analysed using SPIKE2 (C.E.D.) software, with a prewritten analysis program FASTDMEL.TXT. If a histogram of pulse number against IPI length did not show a smooth distribution then subsequent manual editing of events was done using EVENT.TXT to remove spurious events and add events of pulses not marked. All SPIKE2 analysis programs were originally written for use in analysing the original selection experiment (Ritchie and Kyriacou, 1996), and a more detailed description is given in chapter 2.

Temperature has been shown to affect IPI length (Shorey, 1962). Males from the Out stock had been recorded before the first selection experiment over a

temperature range 18-34°C to examine the exact effect of temperature on IPI length, and a significant negative linear correlation had been found, and all IPI values had been adjusted to the expected value for 25°C using the regression coefficient (Ritchie and Kyriacou, 1994). The regression coefficients of mean IPI against temperature for each of the three lines were calculated to test significant deviation and from the regression coefficient found by Ritchie and Kyriacou. The latter was not found and the regression coefficient from Ritchie and Kyriacou (1994) was used to adjust all Mean IPI values to the expected value at 25°C using the equation:-

$$\text{Adjusted IPI} = ((25 - \text{recording temperature}) \times -1.7426) + \text{IPI}$$

3.2.3 Artificial Selection Protocol

Four males of the Long line with the longest IPI were mated to virgin sibling females collected at the same time as the males. Each male was placed in a glass vial containing medium, with a single female for three days, and then both were removed, creating four replicate long lines (Long lines 1-4). This was enough time for insemination and egg laying without larval overcrowding, although larval density was not directly controlled. For the Short line, the four males with the shortest IPI were used to establish four short replicate lines (Short lines 1-4). The use of replicate lines allowed analysis of the influence of drift. Changes due to selection would be consistent across all lines. Differences between lines would be suggestive of genetic drift.

For each of the Long and Short replicate lines, twenty male offspring were isolated upon eclosion over two days. Five minutes courtship song from each of ten males from each line was recorded and digitized, and the mean IPI measured in the same manner as above. Of the twenty males collected for each line, ten were recorded and the remaining ten were held in reserve to replace any males which died, failed to

produce any song, or produced song with an insufficient number of pulses to be used for analysis (less than 50 pulses). All lines were recorded in every session and successive trials used males from stocks sequentially. Virgin females used were collected from a selection line chosen at random each generation.

The males were ranked according to mean IPI, and then the male with the longest IPI of each long replicate line, or shortest IPI of each short replicate line, was mated to a virgin female from the same line to produce the next generation. The male with the second highest IPI (long replicate lines), or shortest IPI (short replicate lines) was also mated to another virgin female from the same line. This second vial was used as a reserve stock, and if the first vial failed to produce adult offspring the offspring from the second vial were used in the next generation. This selection protocol is illustrated in Figure 3.1.

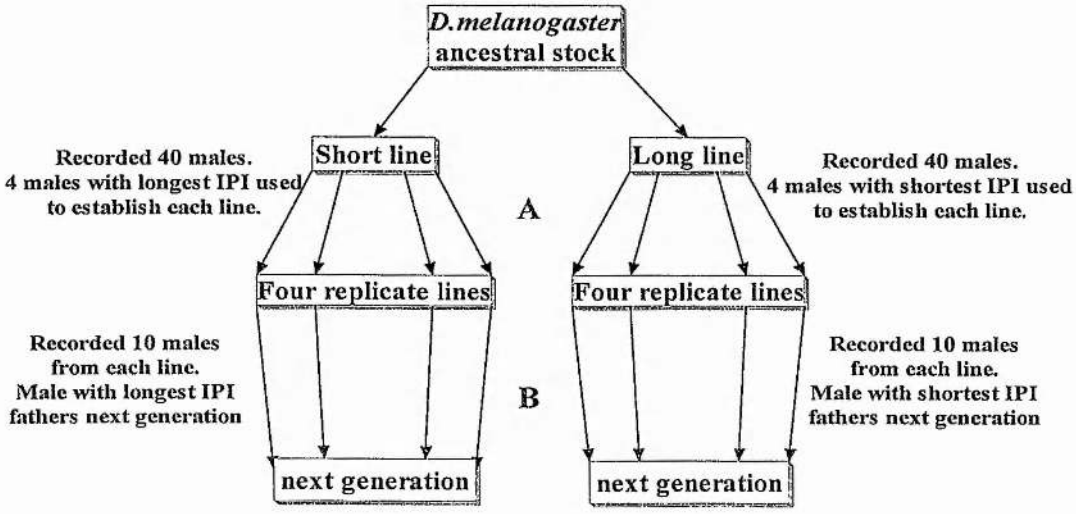


Figure3.1 Bi-directional selection regime for long and short IPI. The Long and Short lines were the result of the initial selection experiment (Ritchie and Kyriacou, 1996). Step **A** created the four replicate lines for each selection regime. Step **B** was repeated each generation during selection.

Selection was continued for twelve generations, with the exception of generation five when insufficient males were produced for recordings. Generation six was established by mass transfer of several offspring of each line to separate fresh vials.

Subsequent to artificial selection all lines were maintained by mass transfer into fresh bottles, as described above. This was continued for ten generations then ten males from each replicate were recorded in the same manner as described previously, with the exception of Long line 1, which had failed to produce enough offspring to be used for recording. After a further 15 generations, ten virgin males for each line were collected and recorded from the replicate lines which had shown the largest response to selection for each selection regime (Short 4, and Long 2) in the same manner, along with ten virgin males from the Out line. Recordings of males from each stock were sequentially interleaved, with all stocks represented during each session. This allowed me to check for convergence in mean IPI across lines.

Control lines were not measured thoughout the selection experiment due to the small time window available for measurement (eighty males measured over two days), this would have reduced the number of individuals from selected lines and/or the number of replicate lines measured. The use of bi-directional selection means that each selection regime acts as a "control" for the other line, and although the standard error of the difference between the two lines is doubled, so is the response as both lines are being selected (see Falconer and MacKay, 1996).

Response to selection of the replicate lines was measured, and the divergence of the two selection regimes fitted with a linear and non-linear equation. The equation used for the non-linear regression was the same as that used in the original selection experiment (Ritchie and Kyriacou, 1996), being a declining exponential function reaching a plateau. The formula used was:-

$$difference = plateau(1\text{-}e^{-k\,gen})$$

(k is a constant and *gen.* is generation). The equation was compared to a linear regression to test if response to selection was linear throughout selection or if response declined. The latter case would suggest fixation of the alleles under artificial selection.

3.2.4 Balancer stocks and chromosome extraction of the selection lines

The two lines Short 4, and Long 2 were used to fix their alleles by crossing with a stock possessing balancers at every chromosome (triple balancer). This was done by taking the line which was to be fixed and crossing the females with triple balancers, following the protocol as outlined in Ashburner (1989).

Balancer stocks contain inversions in the chromosome that prevent crossover in the female over the entire length of the chromosome. This means that sections of the chromosome from the stocks of interest do not become mixed with that of the balancer stock. The balancer chromosome has different recessive lethal mutations on each allele such that progeny will either be homozygous for one of the lethal mutations, or viable heterozygotes. Each balancer is also marked with a dominant phenotypic mutation that allows easy identification for scoring.

3.2.5 Founding triple balancer stocks

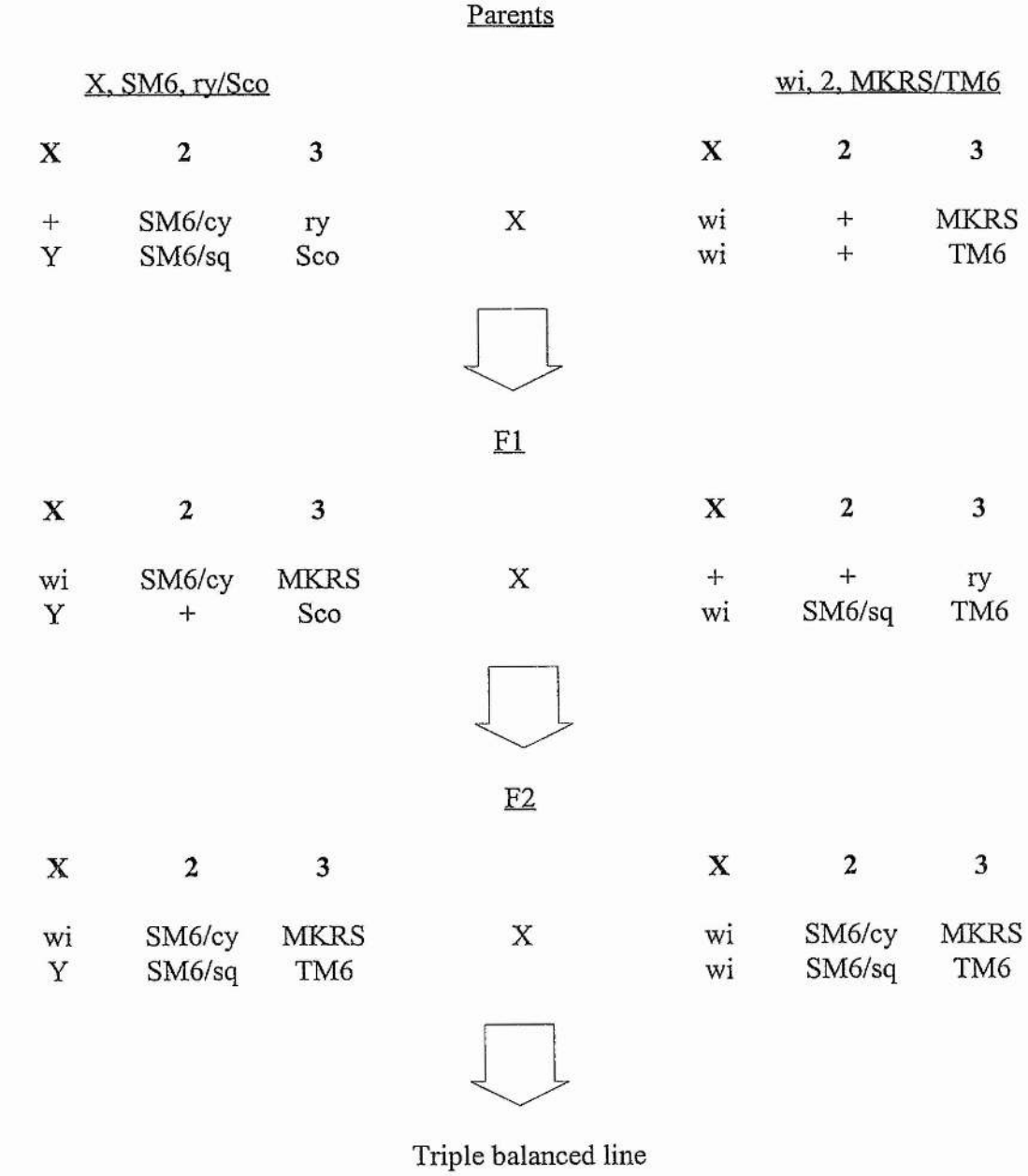
The triple balancer stock was made by crossing two lines of double balancers together. A stock containing the X chromosome balancer **wi**, with the phenotypic markers of white eyes and no ocelli, and the third chromosome **MKRS** and **TM2**, with the phenotypic markers of stubby bristles and round halteres respectively, was crossed with a stock containing the second chromosome balancer **SM6**, with the phenotypic markers of curly wings and short squared wings (**SM6**(cy) and **SM6**(sq)),

and the third chromosome balancer **Sco/ry**, with the phenotypic markers of no ocelli and rosy eyes respectively. The original stocks from which the double balanced stocks were obtained is unknown. The balancer stocks were obtained from Dr. C. P. Kyriacou's Laboratory at Leicester University.

For both stocks, virgin males were collected and separated from females within 24 hours of eclosion under CO_2 anaesthesia. Four males from one stock were placed in a vial with four to five females from the other stock for three days to allow mating and subsequent egg laying.

F1 offspring were collected upon emergence and males containing the balancers **wi**, **SM6**(cy), and **MKRS** and **Sco** were separated and crossed to virgin sibling females with the balancers **wi**, **SM6**(sq), and **ry** and **TM2**.

F2 offspring containing the balancers **wi**, **SM6**(cy) and **SM6**(sq), and **MKRS** plus **TM2**, i.e. triple balanced, were isolated and used for either the chromosome isolation protocol or to found triple balancer lines. Individuals therefore had the phenotype of white eyes (no ocelli); short, squared and curly wings; with stubby bristles and round halteres. The crossing protocol is shown in figure 3.2.



+ = wildtype

Figure 3.2 Genotypes and phenotypes of the triple balancer protocol.

3.2.6 Isogenic Lines

The triple balancer line was crossed to both Long 2 and Short 4, being two of the replicate lines derived from the selection experiment. These replicate lines showed the greatest response to selection for the long and short selection regimes respectively.

Virgin females from the selection line whose chromosomes I wished to make isogenic were isolated upon eclosion. Then a single female was placed together with a balancer male in a vial. Five replicate vials were set up in case insufficient offspring were produced from the first vial.

From the resulting F1 generation, a single virgin male containing the balancers **SM6/sq**, and **TM2** was isolated upon eclosion and backcrossed to two balancer females. Three replicate vials were made.

In the F2 generation five virgin females containing the balancers **wi, SM6/Cy**, and **MKRS** were isolated upon eclosion and mated to four sibling males containing the balancers **SM6/Cy**, and **MKRS**, and therefore the same alleles as their sibling mates, with a replicate vial.

The F3 males produced were therefore isogenic, containing identical alleles at each locus for all chromosomes. These were crossed to virgin sibling females which had one copy of the **wi** X chromosome balancer.

F4 males and females were isolated upon eclosion and those without the **wi** balancer were mated to establish the stocks. The crossing scheme is illustrated in figure 3.3.

The songs from ten virgin F4 males (without the **wi** balancer) from each line were recorded along with ten males from the parental line and ten Out line males, to compare their mean IPI. The long and short lines were recorded during separate sessions with ten Out males recorded each time. Recordings of parental, isogenised, and Out males recorded in the session were sequential. All recordings were done with one day old virgin Out line wingless females. The mean IPI values were adjusted for temperature using the regression value from the original selection experiment (Ritchie

and Kyriacou, 1996). One way ANOVA was used to compare the Short and Long isogenic line with their parental stock and with the simultaneously recorded Out line. Individuals from the short parental line were from generation 31 post selection, and the Long parental line from generation 32.

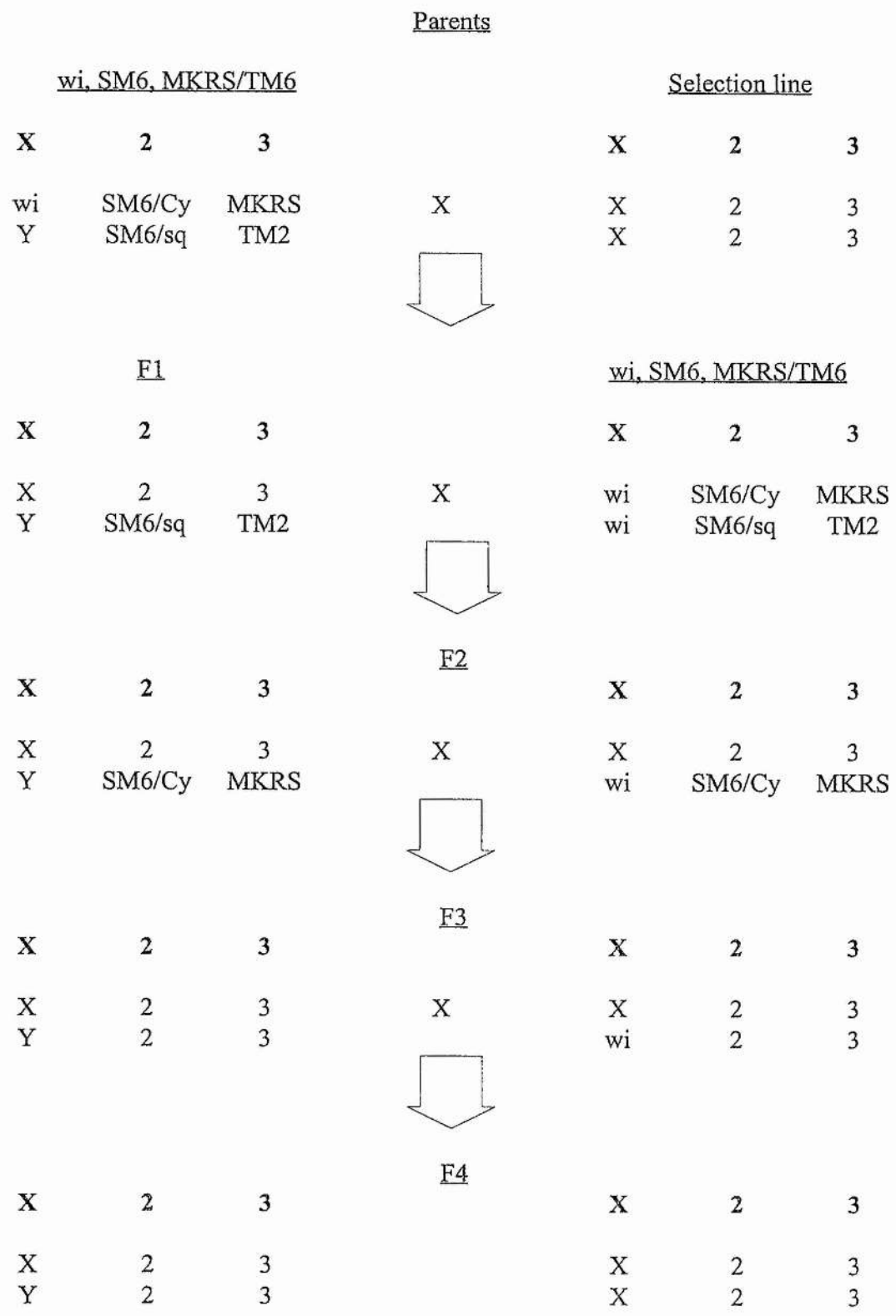


Figure 3.3 Genotypes and phenotypes of chromosome isolation protocol.

3.3 Results

3.3.1 Recordings of stocks from the initial selection lines

Recordings from generation twenty seven and twenty eight after the first selection experiment showed no significant variation between either replicate bottles of the same line, or between different generations.

Mean IPI was significantly correlated with temperature (r^2=0.59, $F_{1,32}$=46.46, P<0.001), but the regression coefficient did not vary significantly from that of the original population (Ritchie and Kyriacou, 1996). Results were therefore corrected for temperature to the values expected at 25°C using the correlation coefficient (1.7426) derived from the original population. Table 3.1 below shows the mean IPI for each line along with their range.

Stock	N	Mean IPI	S.E.	Range
Short line	24	34.47	0.29	31.48-36.89
Out line	23	35.78	0.35	31.05-38.69
Long line	18	37.67	0.50	33.29-41.36

Table 3.1 Mean IPI of the two lines and the control line from the selection experiment by Ritchie and Kyriacou (1996).

The difference between the Long and Short lines was 3.20 msecs, about two standard deviations. The difference in mean IPI between the three lines was significant ($F_{2,62}$=17.47, P<0.001). Fisher's test of individual error rate showed that the Short line was not significantly different from the Out line, but that both were significantly different from the Long line.

The difference in mean IPI of the Short and Long lines at generation 54 after first selection, was found to have fallen to 1.02 msecs from over 4 msecs by the end of

selection, although the difference between the two stocks was still significant ($F_{1,78}$=8.10, P<0.01). This shows a convergence in mean IPI between lines since the end of the initial selection experiment, illustrated in table 3.2. The short line's IPI has remained constant, and the drop in divergence is due to the change in IPI of the long stock. This suggests that selection has acted against males with longer IPI since the end of artificial selection.

Line	Mean IPI		
	End of selection	Generation 27/28	Generation 54
Short	33.50	34.47	33.72
Long	37.98	37.67	34.74
Difference	4.48	3.2	1.02

Table 3.2 Mean IPI of Short and Long lines since the end of initial artificial selection.

3.3.2 Renewed artificial selection

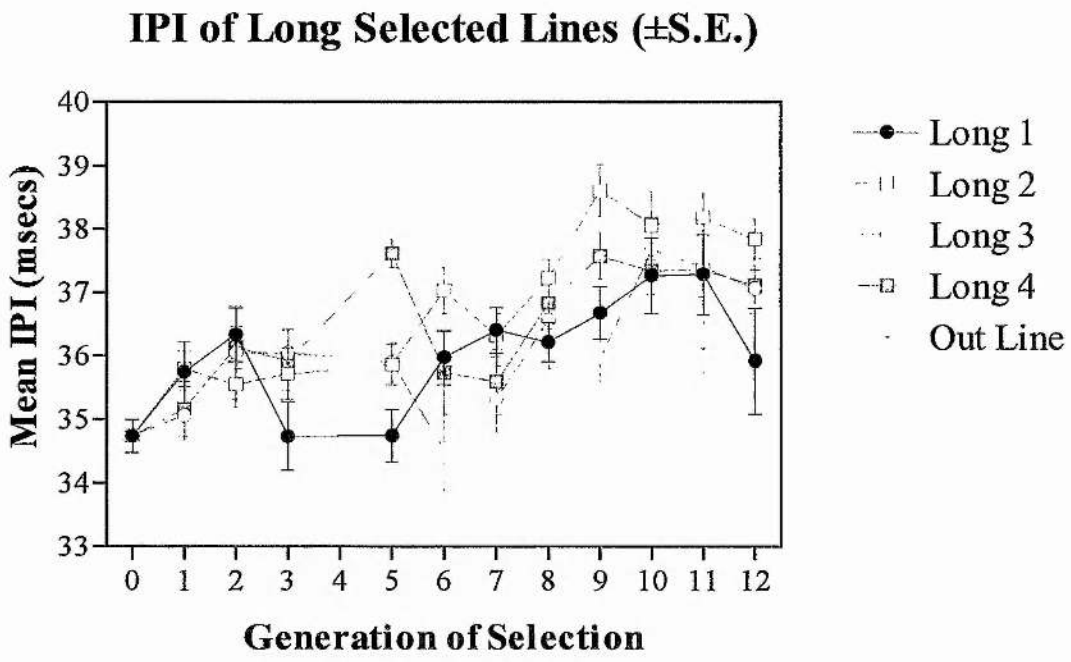


Figure 3.4 Mean IPI of the four replicate long selection lines over the course of the second selection. The Out line measured at the same time as generation 11 is also shown.

Figure 3.4 shows the changes in IPI of the four replicate long lines over the course of selection. Analysis of covariance with generation as the covariate shows that there is a significant difference across lines, as well as a response to selection (see table 3.3). Therefore there is significant difference between lines, with a significant response to selection.

Analysis of Covariance for long regime

Source	DF	ADJ SS	MS	F	P
Covariates	1	191.966	191.966	84.82	<0.0001
long line	3	45.610	15.203	6.72	<0.0001
Error	426	964.170	2.263		
Total	430	1206.682			

Covariate	Coeff	Stdev	t-value	P
long generation	0.1912	0.0208	9.210	<0.0001

Table 3.3 ANCOVA results of the difference in IPI between long lines, with generation as a covariate.

Overall no one line shows a consistent significant difference from the other replicate lines over the course of selection, although Long 2 has the longest value from generation 6 on. The mean difference between lines for each generation was 1.50 msecs (±0.25 S.E.), and never exceeded 3.9 msecs. the long replicate lines and the Out line at generation 11 did not differ significantly.

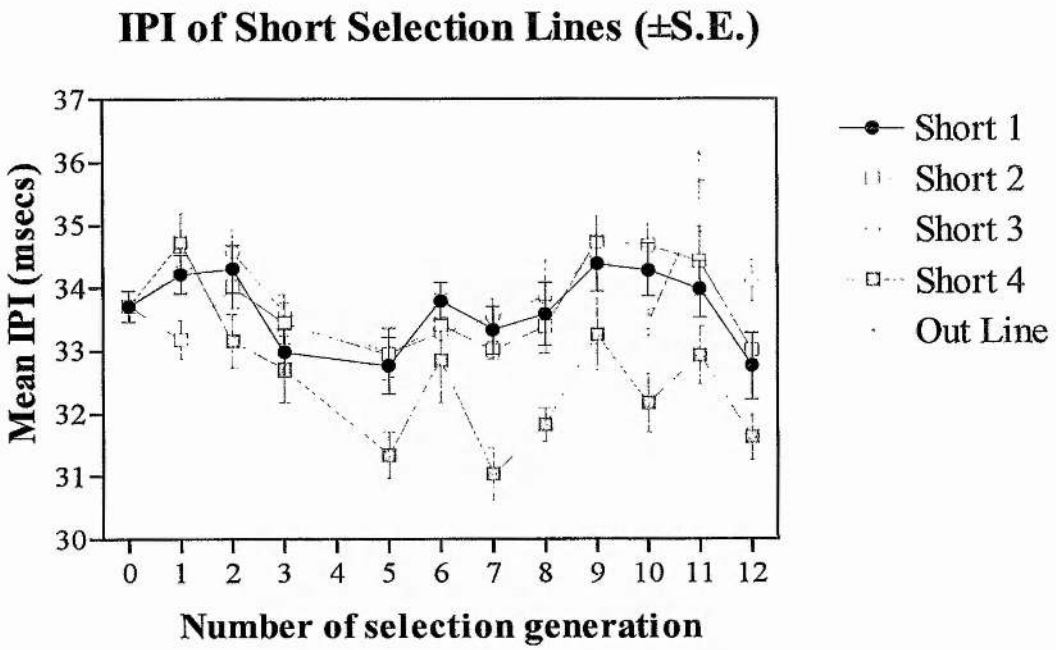


Figure 3.5 Mean IPI of the four replicate short selection lines over the course of selection. The Out line measured at the same time as generation 11 is also shown.

Figure 3.5 show the changes in IPI of the four replicate short lines over the course of selection. Analysis of covariance shows that there is a significant difference across lines, but no response to selection (see table 3.4). There was a significant difference among the short replicate and Out lines at generation 11 ($F_{4,43}$=6.707, P<0.001). However post-hoc testing shows that the Out line is only significantly different from Short 4.

Analysis of Covariance for short regime

Source	DF	ADJ SS	MS	F	P
Covariates	1	0.776	0.776	0.37	0.546
short line	3	136.098	45.366	21.36	<0.0001
Error	430	913.386	2.124		
Total	434	1050.481			

Covariate	Coeff	Stdev	t-value	P
short generation	-0.01200	-0.0198	-0.6046	0.546

Table 3.4 ANCOVA results of the difference in IPI between long lines, with generation as a covariate.

The Short 4 line has a lower mean IPI in all generations from generation 2 onwards and the difference is significant from generation 5, with the exception of generations 6 and 9. The mean difference between lines for each generation was 1.8 msecs (±0.19 S.E.), and never exceeded 2.6 msecs.

It is not possible to draw conclusions using the mean values from different generations, even for the same line due to the large environmental effects on IPI between generations. The values for all replicate lines of both the short and long lines showed large differences between generations outwith that expected from selection. This is most likely due to environmental effects during the development of the males, as temperature was firmly regulated during recordings. Such effects have been previously noted (Ritchie and Kyriacou, 1994; 1996). Therefore comparisons of IPI with that of the Out line at generation 11 is more important for assessing the response across all replicate lines to selection.

The comparison of the number of pulses between the two regimes was significant (T= 3.23, P<0.01, d.f. = 856). It can be seen from the results of the ANCOVA (table 3.6) that there is a significant difference between short lines, and the long lines (table 3.7). The difference is also significant between generations within both regimes. However as is shown by figures 3.8 and 3.9 there is no discernible tends visible between lines or between regimes over the course of selection.

An examination of the mean number of IPI per recording of all individuals from all short and long replicate lines shows that over the first four generations only one line (Long 3) showed a linear regression of pulse number against generation which was significantly different from zero (coefficient=49.29 ± 14.62, $F_{1,3}$=11.37, P<0.05,). Two short lines showed a tendency to increase number of pulses, and two to decrease. The other long lines all showed a tendency to increase in number. It therefore seems very unlikely that mean IPI is strongly correlated with the total number of pulses per song.

Analysis of Covariance for Short lines

Source	DF	ADJ SS	MS	F	P
Covariates	1	2865995	2865995	65.23	0.000
Lines	3	5415668	1805223	41.09	0.000
Error	429	18847960	43935		
Total	433	27262242			

Covariate	Coeff	Stdev	t-value	P
Short Generation	-23.06	2.86	-8.077	0.000

Table 3.6 ANCOVA analysis results for the number of pulses, of songs from different short lines with generations as a covariate.

Analysis of Covariance for all long lines

Source	DF	ADJ SS	MS	F	P
Covariates	1	668193	668193	11.79	0.001
line	3	7094065	2364688	41.73	0.000
Error	426	24139748	56666		
Total	430	31795438			

Covariate	Coeff	Stdev	t-value	P
long generation	-11.28	3.29	-3.434	0.001

Table 3.7 ANCOVA analysis results for the number of pulses, of songs from different long lines with generations as a covariate.

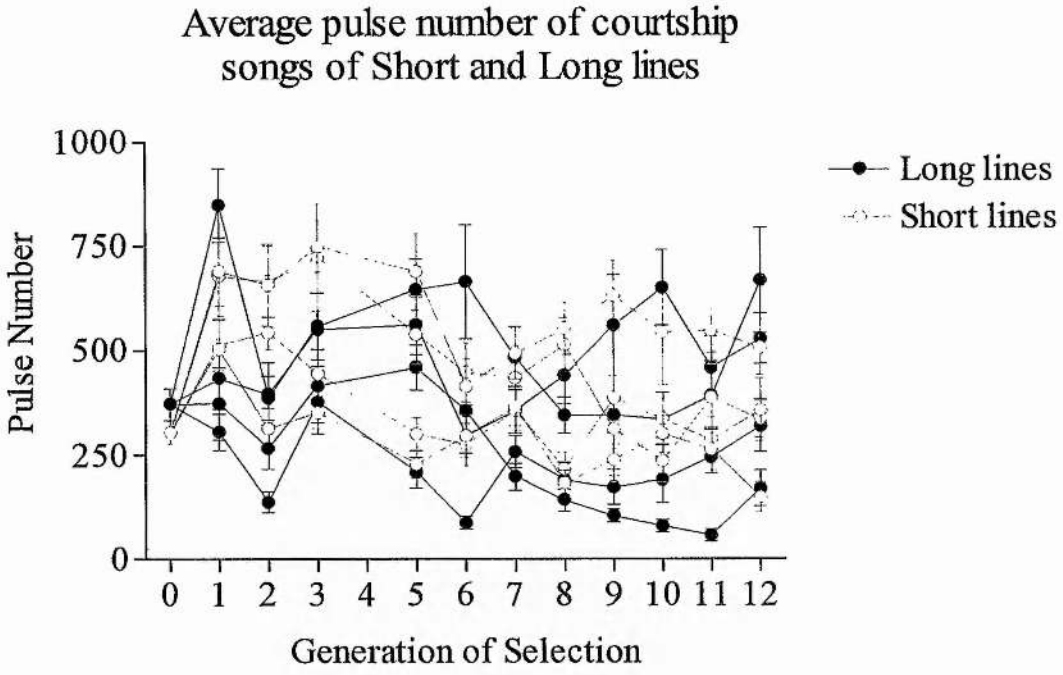


Figure 3.8 Average numbers of pulses from five minutes courtship from males of the different selection lines.

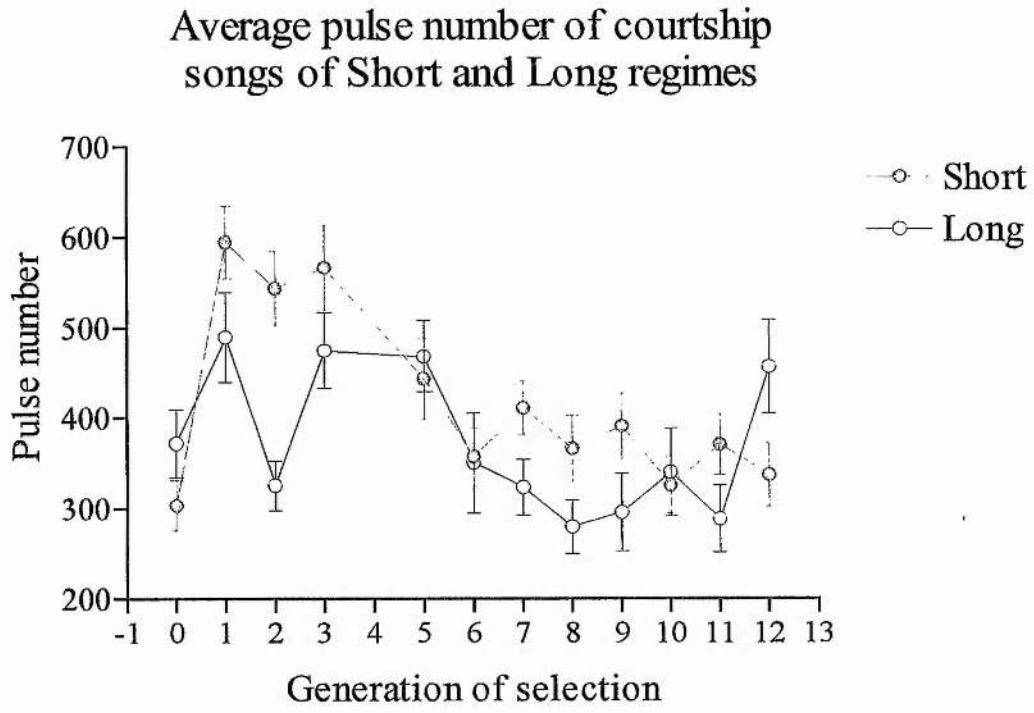


Figure 3.9 Average numbers of pulses from five minutes courtship from males of the different selection regimes.

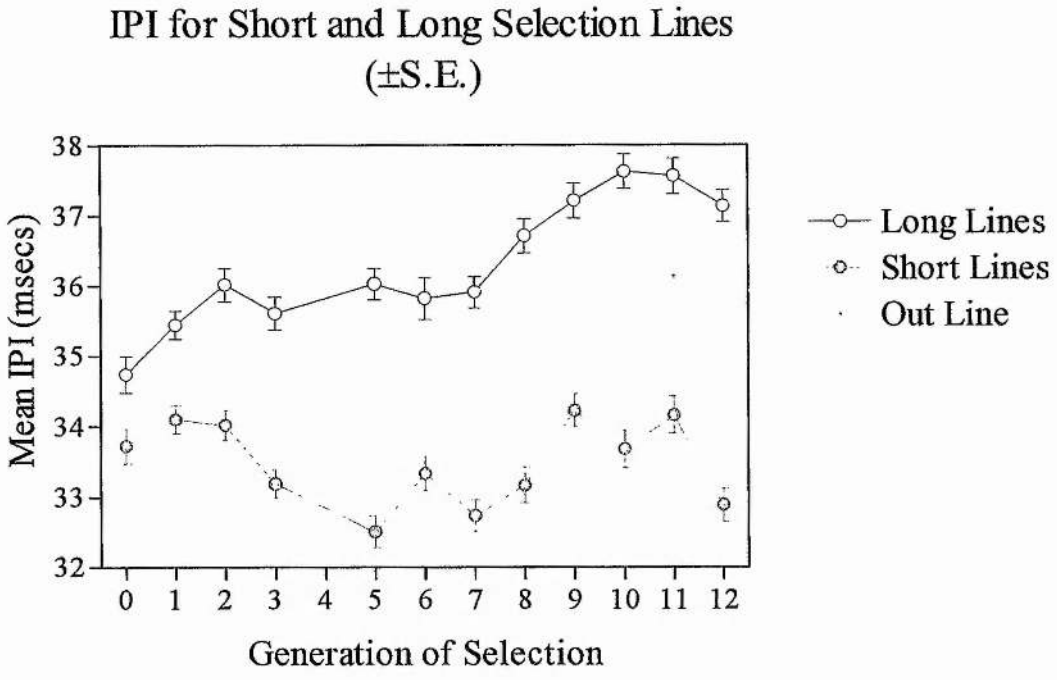


Figure 3.10 The difference between the mean IPI of all individuals from Long and Short replicate lines over the course of selection. The Out line was measured at the same time as generation 11 of selection. Bars are ± standard error.

Figure 3.10 shows the increase in divergence between males from Long and Short regimes over the course of selection, along with males from the Out stock recorded simultaneously with males from generation 11. The recording of Out line males differed from the mean IPI of both the short and long replicate line ($F_{2,85}$=45.50, P<0.0001). However when compared to each long replicate line separately the Out line is not significantly different. The Short replicate lines are significantly different from each other ($F_{3,36}$=4.43, P<0.01), and with the Out line ($F_{4,43}$=6.71, P<0.0001), which has a higher IPI than all short replicate lines.

Figure 3.10 and the statistics for the differences between the regimes seem to imply that the direction of response to selection is greater in the long line. However the differences between the replicate lines from each regime, and the Out line at generation 11 would suggest that it is the short regime that has shown the greater response. It is

response. It is difficult to be sure which is the case when the Out line is only measured at one generation.

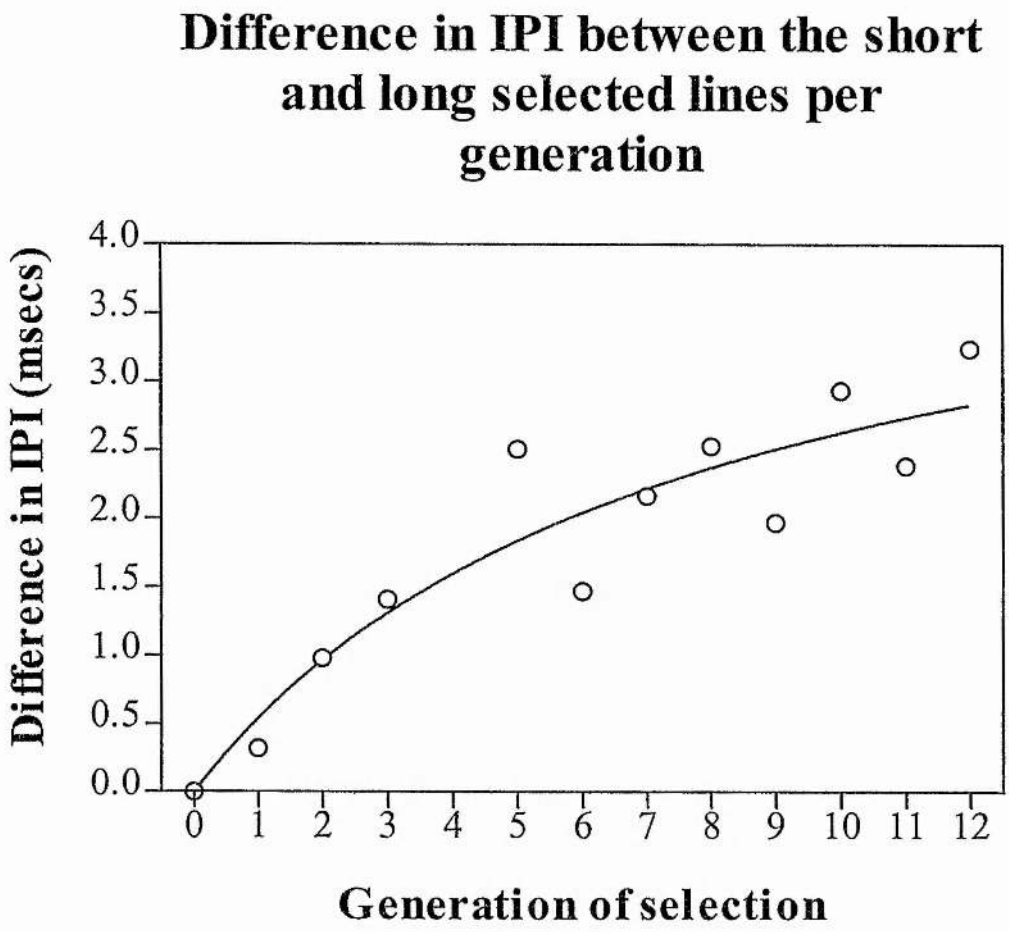


Figure 3.11 shows the change in the difference in IPI between the short and long lines vs. generation, with a non-linear regression line (*difference* = 3.224 (1 - e $^{-0.169\ gen}$).

The difference in mean IPI for all individuals from the long and short replicate lines showed a steady increase until generation five. The difference in mean IPI increased at a steady, but declining rate from generation six onwards (see figure 3.11).

Although large variation was seen in the mean IPI values between generations there was a consistent divergence between the two selection regimes, suggesting that the difference between the two regimes cannot be explained simply by genetic drift.

The difference in IPI over the generations was fitted to a non-linear and a linear regression. The non-linear equation (see figure 3.11) was a significantly better fit than the linear regression ($P<0.05$). This shows that the rate of divergence decreased over the course of selection, possibly due to limited genetic variation. This is similar to the result of the first selection experiment.

There was an initial difference between the long and short lines from the first selection experiment carried out by Ritchie and Kyriacou (1996). It is however, usual to examine the divergence of a character by selection for high and low characteristics from one line (see Falconer and Mackay, 1996). As a difference between the two lines was already present this initial difference was subtracted from the difference between the two selection regimes at each generation to obtain the divergence due to selection from the start of this round of selection.

Line	All Generations	To Generation 5	From Generation 6
Short	0.038±0.092	0.586±0.210	-0.144±0.224
Long	0.416±0.060	0.434±0.194	0.584±0.160
Divergence	0.454±0.068	1.022±0.046	0.440±0.148

Table 3.7 Realized heritabilities ($h^2=R/S$) with standard error values from the regression for both artificial selection regimes, and to the divergence seen between the two regimes.

Table 3.7 shows that the long line showed a greater realized heritability than the short line over the entire selection experiment. This was also the case for response from generation six onwards. However during the first five generations response to selection was greater for the short lines, though not significantly. The divergence over all generations is roughly equal to divergence after generation 6. It suggests that the

latter divergence may be an overestimate given its relatively broad standard error value.

The asymmetry of the total response and the response after generation six agrees with the asymmetrical response seen in the first selection experiment. Asymmetry of response over the entire course of selection is due to reduced response to selection for shorter mean IPI from generation six onwards. From this generation all individuals from the short lines showed virtually no response to selection suggesting that there is a high degree of fixation in the short lines. This is not the case for the long lines. This would broadly agree with a lower heritability for short IPI. However the difference between the regimes and the Out line at generation 11 would seem to disagree with these findings.

The evolvability ($CV_A=100\sqrt{V_A}/mean$) of the trait was 1.64% for short IPI, and 5.68% for Long IPI, derived from the heritability estimates from response to selection over the entire length of this round of selection. Evolvability was 3.33% for short IPI, and 4.38% for Long IPI for the first generation of selection. These values are similar to those seen in the initial selection experiment (Ritchie and Kyriacou, 1996). The low values are due to the low phenotypic variance seen in the parental lines, and because the initial round of artificial selection would have fixed some of the alleles. Even so, there has been a strong response to selection, particularly in the long replicate lines.

3.3.3 Chromosome isolation

There was no significant variation between the short isolation line and Short 4 (table 3.8). Therefore it can be assumed that the chromosome isolation was successful. There was a significant difference in mean IPI between the three lines ($F_{2,27}$=19.99, P<0.0001). Therefore both Short 4 and short isolated line have a significantly different mean IPI to that of the Out line, by post hoc testing. The difference between the Out and both short lines was 3.20 msecs, over two standard deviations. This compares to a difference between Short 4 and Out line mean IPI of 3.21 msecs at generation eleven of selection. The mean values and ranges of mean IPI of the three lines shown in table 3.4 below.

Stock	N	Mean IPI	S.E.	Range
Short 4	10	29.98	0.43	27.78 - 31.52
Short isolated	10	29.89	0.37	28.32 - 31.21
Out line	10	33.18	0.45	30.81 - 34.88

Table 3.8 Mean IPI of Short 4, the short isogenic line after isogenisation, and the ancestral Out line.

There was a significant difference between Long 2, long isogenised and Out lines ($F_{2,13}$=25.28, P<0.001), with all lines being significantly different from each other by post hoc testing. The difference between the Out and Long 2 was 3.42 msecs (just over 3 standard deviations), and between the Long isolated line and the Out line was 5.14 msecs (just over 4.5 standard deviations). This compares to a difference between Long 2 and Out line mean IPI of 2.06 msecs at generation eleven of selection. However while the mean IPI of Long 2 and long isolated line are both significantly higher than the Out stock, the long isolated line's mean IPI is higher than

Long 2. The mean values and range of mean IPI of the three lines is shown in table 3.9 below.

Stock	N	Mean IPI	S.E.	Range
Long 2	6	35.45	0.51	34.20 - 37.08
Long isolated	6	37.47	0.46	35.58 - 38.53
Out line	4	32.03	0.43	31.05 - 33.00

Table 3.9 Mean IPI corrected for temperature of Long 2, the long isogenic line after isogenisation, and the ancestral Out line.

The isolation of an isogenic line from Long 2 has therefore been successful. It seems highly unlikely that the swap was contaminated or incorrectly performed, as this would result in a stock with higher mean IPI than the Long parent stock. The difference between the mean IPI of the isogenised line and the mean IPI of the Out line is greater than that seen between the two selection regimes by the end of selection ($t=8.18$, d.f.=7, $P<0.0001$). The more likely explanation is that the long isogenisation line has genes responsible for mean IPI that are at the high end of the range in the selected stock, and that the significant difference in mean IPI is due to type I error. It should also be noted that the sample sizes of all three populations are small, which was due to limited numbers of emerging offspring.

3.4 Discussion

There is a large response to selection despite high stereotypy and low evolvability of the initial populations and also the low heritability by father-son regressions of the original stock before selection (Ritchie *et al*, 1994, Ritchie and Kyriacou, 1996, this study). The response in the long lines was significant even though there has been significant drift between replicate lines. Drift was also seen in the short lines and response to selection for shorter IPI has not produced a significant response. It was, however, significantly different from the Out line at generation 11, This was a rather surprising result, but given the asymmetry in response seen the first round of artificial selection, the asymmetry shown in this round of selection is real. Therefore the asymmetry of response seen in the initial experiment is real and not a result of drift. There is obviously still additive variation for these traits even after one round of artificial selection. It would seem that the low evolvability within the species does not preclude rapid change in IPI, which could contribute, possibly to a large degree, to sexual isolation of nascent species. Indeed IPI is highly variable between species within the *Drosophila willistoni* group, and greater than the divergence in morphology (Ritchie and Gleason, 1994), and high variation in signals between closely related species is thought to be a common feature of singing insects (Henry, 1994).

Ritchie and Kyriacou (1996) suggested that selection for shorter IPI may be selection for increased sexual activity. They concluded that the selection for longer IPI may have resulted in a drop in pulse number. This drop in pulse number and an asymmetry of response to selection, higher for long IPI, means that IPI may have some association with reduced sexual activity. However the lack of replicate lines in the initial experiment by Ritchie and Kyriacou (1996) did not rule out the possibility that the change in pulse number seen in the long line was due to drift.

The number of pulses varied in both regimes across lines and across generation. However the change was not consistent, or associated with the change in

IPI. It would therefore seem likely that change in number of pulses over the course of selection was mainly due to drift.

An asymmetry of response to selection was also seen in this experiment. Such asymmetry of response is usually associated with a character under directional selection. Although selection for a shorter mean IPI is not directly linked to pulse number, it may be linked to other factors affecting fitness.

It seems likely that as females perceive pulses as sequential movements of the aristae, a burst of set length will contain more pulses in males with shorter IPI which would result in an increase in the number of neural responses sent from the aristae, providing that the neurone signals once for each deflection of the arista. The same increase in stimuli over a longer period of time could be obtained by increasing the total number of pulses per burst over that time while maintaining the IPI. The female may have a threshold, and need a set number of stimuli from the aristae to induce sexual receptivity. A decrease in time taken for this threshold to be reached could be achieved either through an increase in total number of pulses over a set time while maintaining the IPI, or by a shorter IPI while maintaining the burst length.

Frankham (1990) suggests that over the relatively short number of generations over which artificial selection pressure is maintained, the genes being selected for would need to be of major effect to produce the response seen in artificial selection experiments. The initial equal response to the two selection regimes seen here could be due to selection in the short lines acting on genes of relatively large effect at the beginning of selection, which rapidly become fixed around the middle of selection. The steady rate of response of the long replicate lines throughout selection means that the genes being selected upon are not becoming fixed. This may be due a larger number of genes of lesser individual effect producing a similar level of response, with each gene taking longer to come to fixation, than those responding to selection for shorter IPI. Alternatively the number and amplitude of effect of the genes responding in both regimes may be similar, but with those in the short lines occurring at a higher frequency in the original Pietrastornina population. If over the recent evolutionary

history of the species female preference had been consistently directional for shorter song, then the genes causing a shorter IPI should have been selected for. The maintenance of heterozygosity for these alleles may be due to some negative pleiotropic effects of homozygous alleles for shorter song, heterozygous advantage, a high level of mutation at those alleles as suggested by Pomiankowski and Møller (1995), or the large environmental effects on mean IPI.

The difference between Long 3 and Short 4, and the Out line has been maintained during relaxation of selection pressure and chromosome isolation, reinforcing the idea of fixation of alleles in one or both lines during selection reduced the rate of divergence over the course of selection. As the lines used were those showing the most extreme phenotype it is not suprising that they would show a high level of fixation, thus maintaining the difference seen by the end of selection. The isogenization of the two lines means that the difference in mean IPI between lines established by selection will not converge and can be used for future studies into the genetic basis of the difference.

The high level of divergence seen at the start of selection contrasts with the levelling off of the response to selection seen in the initial round of selection. This disparity may be due to recombination among loci allowing renewed response to selection. It is also possible that there were mutations at the loci responsible, although this would seem unlikely given the limited number of generations between the two rounds of selection.

CHAPTER 4: FEMALE MATING SPEEDS OF SELECTED LINES

4.1 Introduction

It has been suggested that signal and receiver systems must maintain co-ordination during the evolution of the system. The "Genetic Coupling" hypothesis suggests control of both by closely linked genes or the same gene (Alexander, 1962). Although this is an attractive theory, the evidence for it is scant and such a system would have to be controlled by a few genes of major effect (Butlin and Ritchie, 1989). This contrasts with the theory of more independent evolution such as sensory exploitation where a pre-existing (female) preference provides selective pressure on the male trait (Ryan, 1990; Ryan *et al.*, 1990). Studies on fish and frogs have highlighted cases where the female preference for a male trait is not congruent with the trait (Ryan and Wagner, 1987; Basolo, 1990, 1995; Ryan and Rand, 1993).

Male stalk-eyed flies, *Cyrtodiopsis dalmanni*, display a huge eye-span and females prefer males with the longest span (Burkhardt and de la Motte, 1988). After bi-directional selection on stalk-eyed flies for eye-span length Wilkinson and Reillo (1994) found a correlated response in female preference. In choice tests females from short eye-span selected lines preferred short eye-span males, though long line preferences were unchanged.

Female guppies, *Poecilia reticulata*, prefer males with higher amounts of orange tail pigment, and the female preference varies between different populations of the species (Houde, 1987; 1988). This pigment makes them more conspicuous to predators, and it has been shown that females in areas with lower predation risk preferred more brightly coloured males (Stoner and Breden, 1988, Houde and Endler, 1990). Two studies have selected for the male trait of orange colour in guppies. Houde (1994) found that female preference of the high line females was significantly higher than the low lines for the first two generations but this difference was lost by the third. Breden and Hornaday (1994) found no difference in preference after five generations

of selection. Pomainkowsi and Sheridan's (1994) review of both papers suggested that the lack of preference seen by Breden and Hornaday could be due to males being assigned to females during selection instead of females being allowed to choose a mate during the course of selection as Houde found. This is because most colour genes are Y linked so covariance will decrease by half each generation.

A change in a male trait should be accompanied by a corresponding shift in the female preference if the two are genetically linked as suggested by Alexander (1962). Chapter three of this thesis describes twelve generations of artificial selection on *D. melanogaster* for long and short mean IPI. Selection resulted in a 4.3 msecs difference between the mean IPI of all Short and Long lines. The difference between the most divergent individual lines was 6.2 msecs. The Long replicate lines IPI value was close to that seen in *D. erecta* and *D. orena*. These two species are the most distantly related species to *D. melanogaster* within the *melanogaster* subgroup and are thought to have diverged some 2.5 million years ago. There has been ample time for the signals of the two species to diverge from that seen in *D. melanogaster* (Lachaise *et al.* 1988). The long lines' value was similar to that seen by Ritchie and Kyriacou (1996) during previous selection on the same stocks. Studies of female preference would be possible with such a large difference in IPI.

It would seem unlikely that change in male IPI could cause a comparable shift in the response distribution of females, as IPI seems to be polygenically controlled within *D. melanogaster* (Cowling and Burnet, 1981; Ritchie and Kyriacou, 1996), and between species within the *melanogaster* complex (Chapter 3). Genes affecting female preference would have to be linked to all genes controlling IPI thoughout the genome. However, weak changes in female preference for mean IPI may still be seen if there is linkage between one or more of the loci responsible for mean IPI and loci responsible for female preference. This should be consistent across the replicate lines for each selection regime. Changes due to random genetic drift would be very unlikely to be consistent across replicate lines.

I wished to study the response of females of each replicate line to the males from all replicate lines of both selection regimes, and compare these results to that seen in the ancestral (Pietrastornina) line. Mating speed is used as a measure of female preference. The higher rate of matings by females with males from one line over males from another line is therefore most likely to be due to female preference in the former line. The effect of male preference cannot be ruled out altogether, but should be limited through experimental design.

Any change in the distribution of the preference for mean IPI by females of the selected stocks in the same direction as selection may suggest covariance of the loci for mean IPI and female preference for mean IPI. This would mean that any shift in the mean IPI would cause a response in female preference resulting in maintenance of co-ordination between signal and preference means. If there is no linkage then the female preference mean of the population would have to respond independently in order re-establish co-ordination of the signal and preference mean. If it did not change there would remain a mismatch of signal mean and preference mean. Females would retain a preference for the original population mean IPI.

Female preference was tested for any difference in response to males from different lines, and to males from the two different selection regimes. This was to examine if females consistently preferred males from any one line or males from any one regime. A consistently larger mating rate of females across all lines to males of their own line and own regime would suggest the possibility of covariance between mean IPI and female preference for mean IPI.

4.2 Methods.

4.2.1 Mating in replicate lines.

The stocks used were Short 1, Short 2, Short 4, and Long 1, Long 2, Long 3, Long 4. These are replicate lines created by artificial selection for short and long mean IPI (Chapter 3). Each stock was maintained in two replicate half pint milk bottles containing standard medium (chapter 2) in an incubator on a 12/12 hour light/dark cycle at 25^{o}C. Further generations were established by mass transfer of thirty to forty individual adults to new bottles containing fresh media, although exact numbers were not measured. The line Short 3 was not used in this experiment as sufficient numbers were not available.

4.2.2 Female mating rate.

Four generations after the end of artificial selection virgin adults from each replicate line were collected upon eclosion and sexed. Males and females were kept separately in 30 ml glass vials containing medium for three to four days. Anaesthesia was by CO_2 during collections and transfer of flies. After two days, fifteen males, and fifteen females of a single replicate line were anaesthetised and placed into a transparent perspex fly mating chamber, illustrated in figure 4.1. Sexes were separated by a gauze barrier. The flies were allowed one minute to settle and recover. To start the mating trial the barrier was then withdrawn, and the sexes allowed to mix. The number of pairs copulating was recorded for the following 15 minutes at two and a half minute intervals. Trials were repeated for each combination of males and females from each replicate line, with five replicate trials per combination. All males and females were used for only one trial unless no matings occurred during the trial, when individuals were anaesthetised, separated by sex and reused with a gap of at least one

intervening trial to allow individuals to recover from the immediate effects of CO_2. The sequence of trials was randomised for combination, and were performed during the twelve hour light period of the diurnal light dark cycle. Trials were recorded over five generations (Generations four to eight after the end of selection). Temperature was held at 25±1°C throughout all trials.

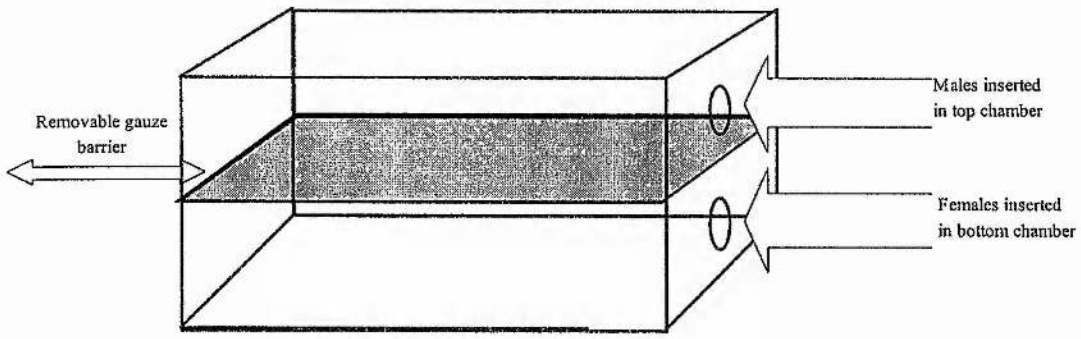


Figure 4.1 Perspex fly mating chamber (5 cm width x 5 cm length x 3 cm height).

4.2.3 Data analysis

The data was analysed by two-way ANOVA using MINTAB to examine the variation in numbers of females mated to males. Trials were grouped by trial and according to the replicate line of the females and males used, and females from the same selection regime with males from the same selection regime. If mean IPI is the principal agent of preference then a difference should be seen in female preference for males from the different regimes, even when response to the individual replicate lines are different, perhaps due to vigour effects between lines. Factors that affect response in the same direction in all lines will be emphasised by grouping, but antagonistic factors between replicate lines will not.

Also, the data were fitted with a non-linear regression to determine rate of mating and to compare mating success of males over the course of the trial. The equation assumes that the number of pairs mating would eventually reach a plateau that can be outwith the time of the trial, and equal to the number of pairs used in the trial. This equation fits the data well, and was a better fit than a straight line and the same non-linear regression with a variable plateau for all data sets. The biological justification for using this equation is considered in the discussion.

The equation for the curve is: $Y = 15 * X/(Kd + X)$, being a hyperbola. Kd is the time taken until half of all individuals have mated. Kd is inversely proportional to the rate of mating. Thus a higher Kd means that females were mating less rapidly with males, so showing a higher latency before mating. This equation was fitted by the Graphpad Prism program.

The Kd values of females and males from the different regimes were compared by two-way ANOVA using Graphpad Prism, and females of each line with males from the different replicate lines by one-way ANOVA.

4.2.4 Female mating rate trials using winged and muted males.

A consistent tendency for females to mate with males from lines Short 2 and Long 3 was found. Also females from these two lines preferred males from their own lines to those from the other line. The difference in mean IPI between the Short 2 stock and the Pietrastornina stock was over 1.7 msecs, and over 1.2 msecs between Long 3 and Pietrastornina lines at generation eleven of selection on the long and short lines (see chapter 3). These lines were selected for further examination to study the basis of any assortative mating, by the use of muted males to determine the extent that courtship song played in the discrimination of females.

Mating trials for winged males were performed over fifteen minutes in the same manner as before, and were measured over thirty minutes for wingless males,

which had been muted by cutting the wings back to the base of attachment without damage to the thorax. Seven replicate trials using winged males were performed with eleven replicate trials using wingless males. The data was analysed by one way ANOVA.

4.2.5 Pietrastornina line female mating trials.

The mating speeds of the Pietrastornina line females with the males from the same stock, from Short 2, and Long 3 lines were measured. Males from all three lines and females from the Pietrastornina line were collected on the same days. Males of the selection lines were from four to nine generations after the end of selection.

Female mating speed was measured using the same method as described in 4.2.3 with six replicates per combination. All males and females were used only once unless no matings occurred in the trial, in which case individuals were separated by sex and reused after at least one intervening trial. Males were not reused with the same females. All trials were interleaved by male line, with males from all lines being tested in the same session. Two day old flies were used in all trials.

The data were fitted with the same non-linear regression as before, to test for differences in mating speed of Pietrastornina females with males of each line. The numbers of pairs mating and the Kd was analysed by one-way ANOVA.

4.3 Results.

4.3.1 Relative female mating rates in replicate lines.

Long 1 males were highly unsuccessful at achieving mating within the fifteen minute period of the trial, with only one mating from thirty five trials. During the trials these males were observed to be much less active than males from all other lines. They were unresponsive to the presence of females, making little or no attempt to mate, or indeed move at all thoughout the trial period. This was either because they were unresponsive to the presence of females, failing to recognise them as potential mates, or extremely inviable in general. The former seems unlikely to be the case as males from all other long and short stocks showed brief abortive courtship even with males they have newly encountered (pers. observ.) and this was not observed for the Long 1 males. It is more likely that the stock was unfit as maintenance of the stock was difficult. Also attempts to record the song of Long 1 males after the trials were unsuccessful as males showed no propensity to court, or indeed move around the chamber during attempted recordings. Females of the Long 1 line did mate at similar rates to those from other lines. However, it was necessary to exclude Long 1 females from the analysis in order to balance the two-way ANOVA.

Source of variance	DF	SS	MS	F	P
male regime	1	39.200	39.200	5.24	0.023
male line (male regime)	4	195.333	48.833	6.53	0.000
female regime	1	16.200	16.200	2.17	0.143
female line (female regime)	4	89.933	22.483	3.01	0.020
male regime * female regime	1	0.022	0.022	0.00	0.957
Error	168	1256.511	7.479		
Total	179	1597.200			

Table 4.1 Analysis of variance results table from MINITAB for difference in female mating speed crossed with male regime, with male lines nested within male regime.

The results of the ANOVA are shown in table 4.1. There is a significant difference in the level of mating for males from the different regimes. The difference becomes highly significant when male lines are nested within regimes. The non-significant difference between the levels of mating between females grouped by regime shows that artificial selection has not affected the average response to males by females of the two regimes. The significant difference seen when female lines are nested within regimes explains much the non-significant difference between female regimes. It can be seen that in the interaction between male and female regime, the change in mating speed was not significant.

The result would suggest that difference in preference for males over the range of mean IPI shown by the replicate lines tested does not seem to be a determinant of mate choice in females according to their regime. The difference in levels of mating seen between regimes is due to differences between lines, and these differences are not due to the differences in IPI of the two regimes.

Source of Variation	Df	Sum-of-squares	Mean square	F	P-value
male	1	49180	49180	19.71	<0.0001
female	1	14580	14580	5.845	0.0158
Interaction	1	4764	4764	1.91	0.1673
Residual	1252	3124000	2495		
Total	1255	3192000			

Table 4.2 Results table of two-way ANOVA of Kd values for non-linear regressions of mating of females and males from the two regimes.

Figure 4.2 shows the average number of matings for females and males grouped by regime with the line fitted by non-linear equation for each. Figure 4.3 shows the Kd values from these equations. In figures 4.2 and table 4.2 it can be seen that short males show a higher rate of mating than long males for each female regime, and overall. There is a significant difference in the rate of mating between female regimes and male regimes. Long females show a faster rate of mating, but both female regimes prefer short regime males. This does not show assortative matings for the long females with long males. It is likely that females from both lines show a mating preference for males with shorter IPI. Although the trend is not significant, discrimination against long males may be exaggerated in females whose sibling males display shorter IPI as shown in figure 4.3.

Female Lines	Short male lines	Long male lines
Long 2	$F_{2,99}$=6.383, P<0.01	$F_{2,99}$=8.987, P<0.001
Long 3	$F_{2,99}$=4.720, P<0.05	$F_{2,99}$=6.626, P<0.01
Long 4	$F_{2,99}$=18.23, P<0.0001	$F_{2,99}$=7.284, P<0.0001
Short 1	$F_{2,99}$=6.027, P<0.005	$F_{2,99}$=8.575, P<0.001
Short 2	$F_{2,99}$=17.23, P<0.0001	$F_{2,99}$=8.418, P<0.0001
Short 4	n.s.	$F_{2,99}$=6.284, P<0.005

Table 4.3 Results from one-way ANOVA for the difference in Kd of mating rates for females from each line mating with males from short lines and long lines respectively.

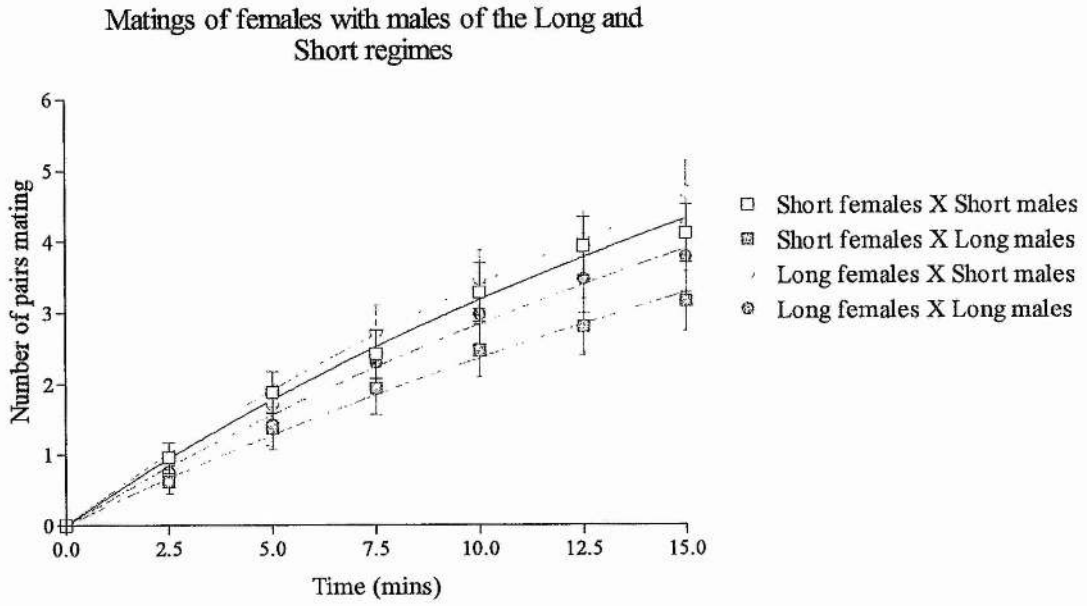


Figure 4.2 Average number mating of females from each regime with males from each regime (with standard errors).

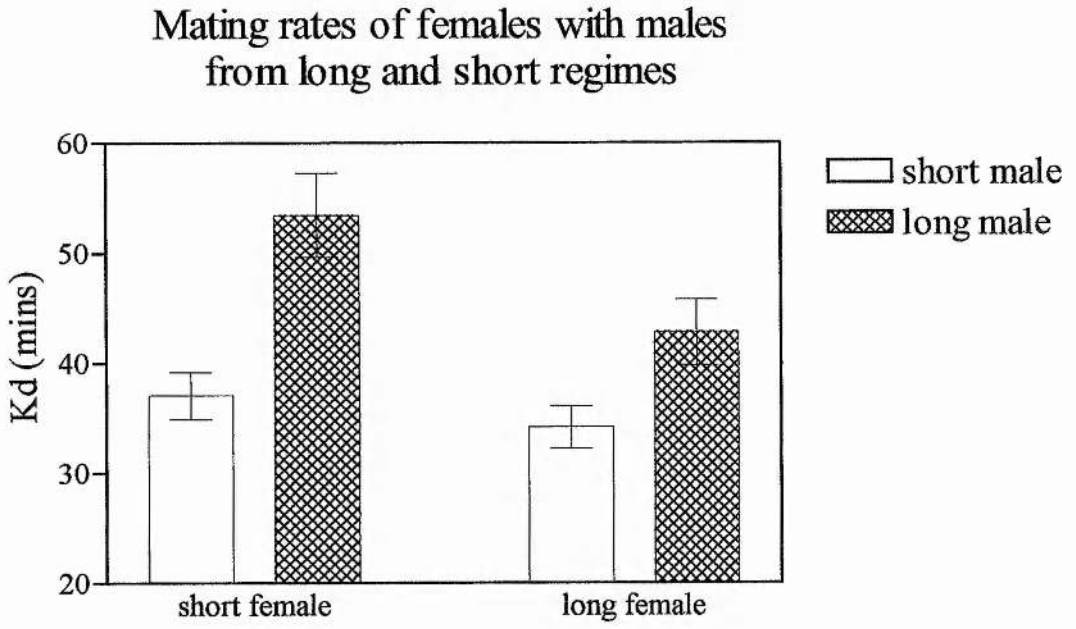


Figure 4.3 Kd values for females to males of each regime (with standard errors).

Figures 4.4, 4.6, 4.8, 4.10, 4.12, and 4.14 show numbers mating over the trial period for each female line, and are grouped by male line. They are shown without error bars for clarity. Figures 4.5, 4.7, 4.9, 4.11, 4.13, and 4.15 show the Kd values of the non-linear equation fitted to the numbers mating for each female line.

Over all long female lines, response to males from the short and long replicate lines were highly interleaved with no significant difference among lines. Table 4.3 gives details of the differences in Kd of males from the same regime but different lines. The large degree of variation between lines confirms that the difference between regimes is greatly affected by the origin of the male line.

It can be seen that the comparison of different Kd values gives a higher resolution in the differentiation of mating levels between regimes and lines than the total number of matings achieved by the end of the trial.

The interleaving of mating speeds of Long 2 females to the lines of the two different regimes in figure 4.4 is typical of the pattern seen in all female lines. The distribution of Kd values of Long 2 females to males of the different lines shown in figure 4.5 again reflects the general aspect of Kd values in other female lines.

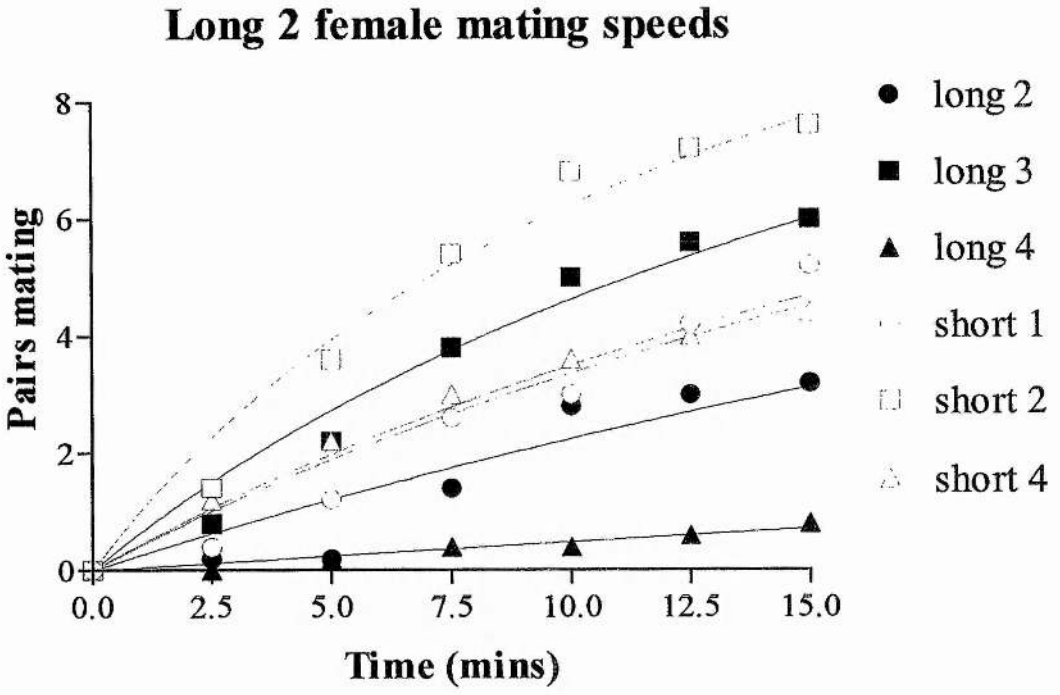


Figure 4.4 Average number of matings of Long 2 females with males from each of the replicate lines fitted with the non-linear regression.

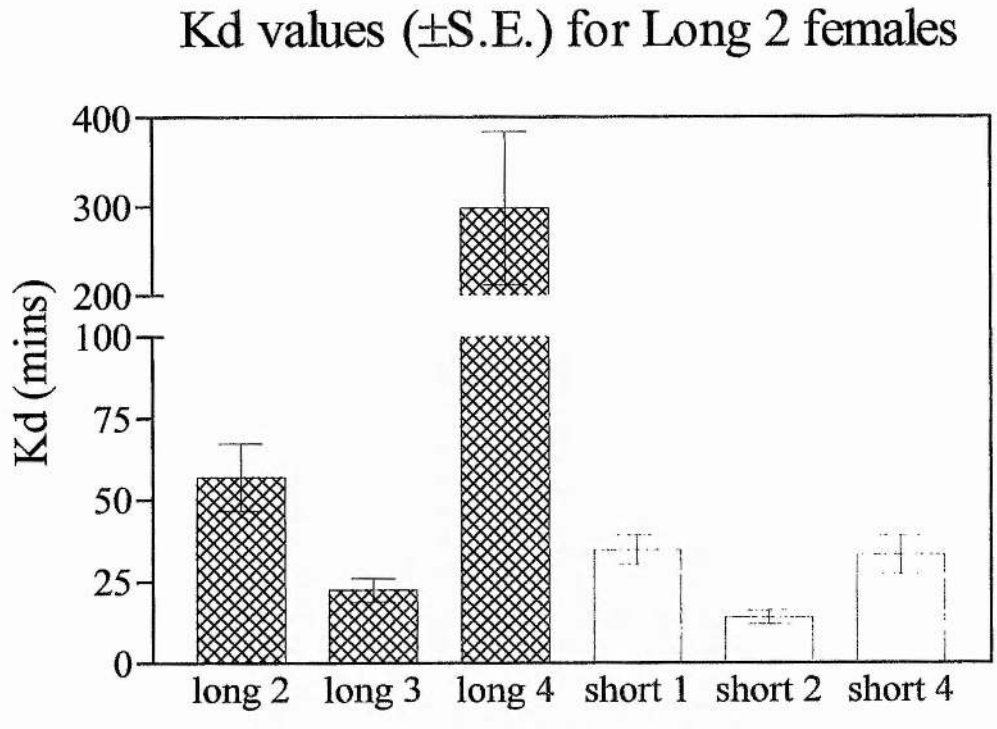


Figure 4.5 Kd values for Long 2 females to males of each replicate lines.

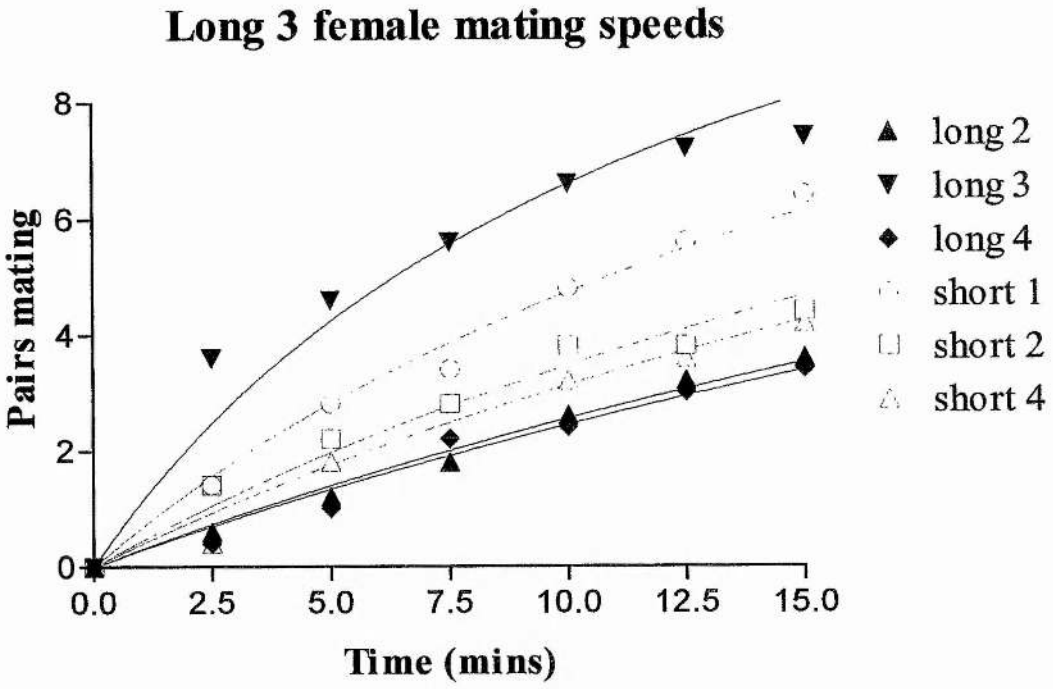


Figure 4.6 Average number of matings of Long 3 females with males from each of the replicate lines fitted with the non-linear regression.

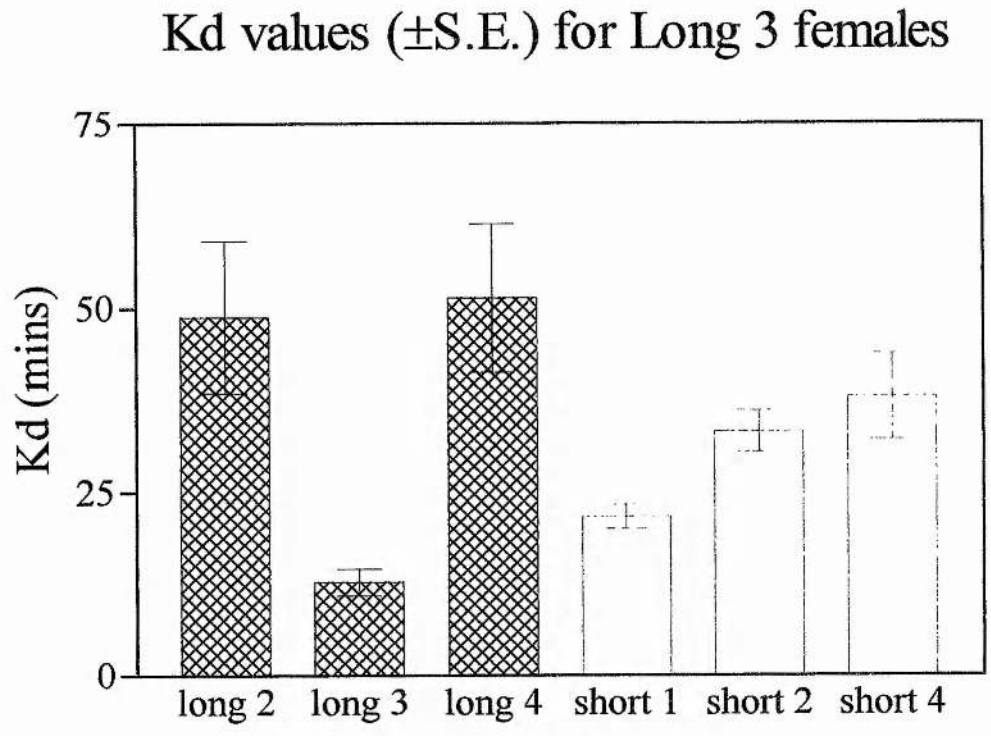


Figure 4.7 Kd values for Long 3 females to males of each replicate lines.

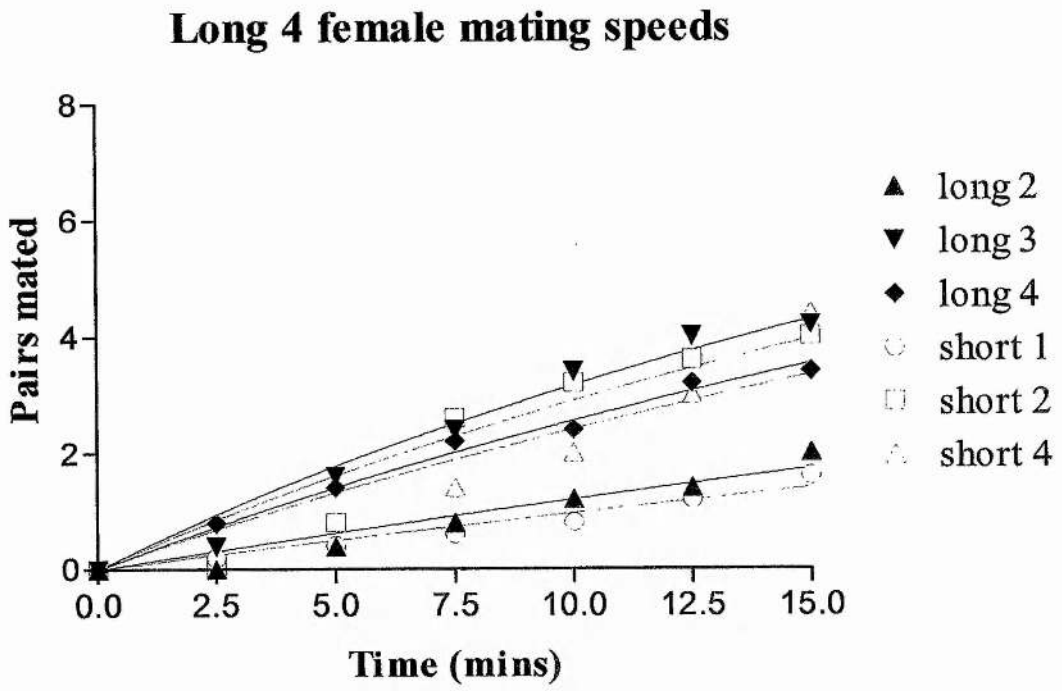


Figure 4.8 Average number of matings of Long 4 females with males from each of the replicate lines fitted with the non-linear regression.

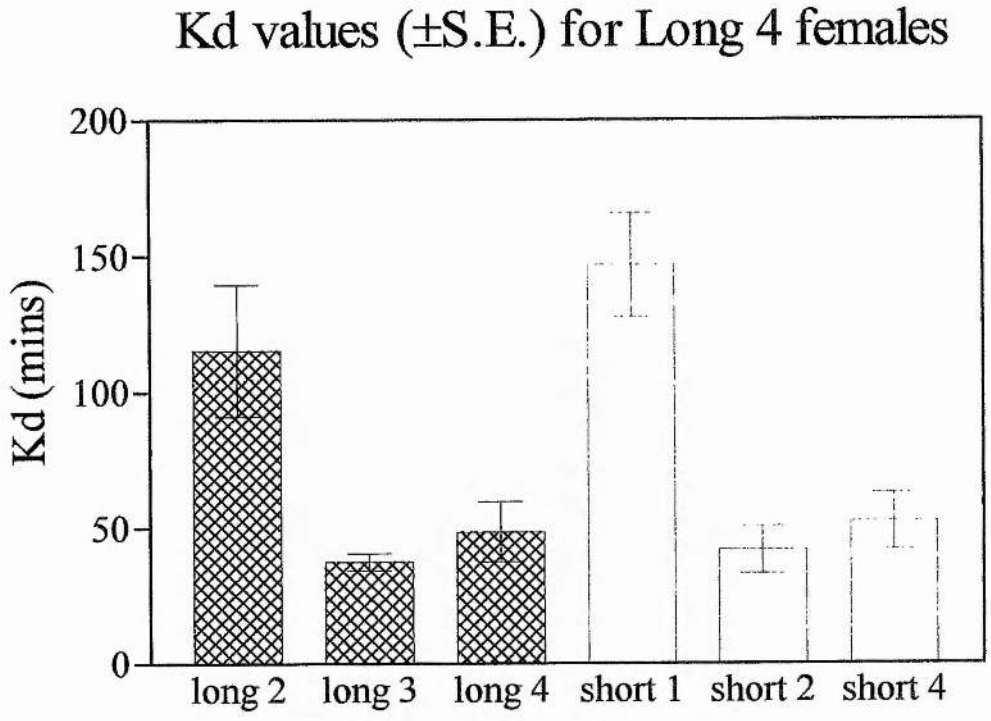


Figure 4.9 Kd values for Long 4 females to males of each replicate lines.

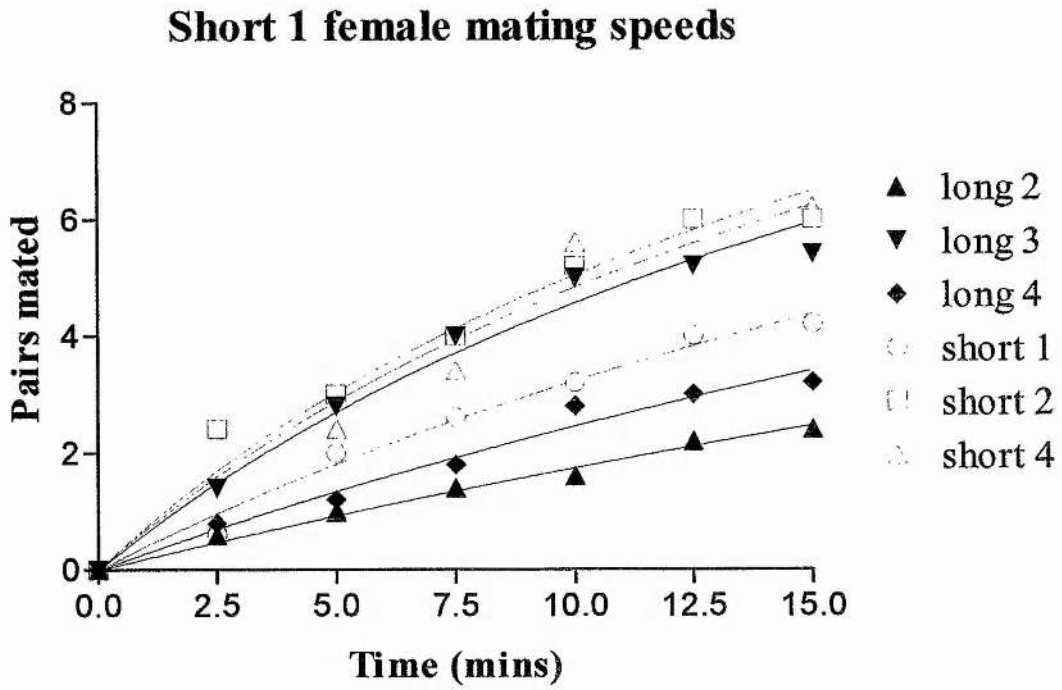


Figure 4.10 Average number of matings of Short 1 females with males from each of the replicate lines fitted with the non-linear regression.

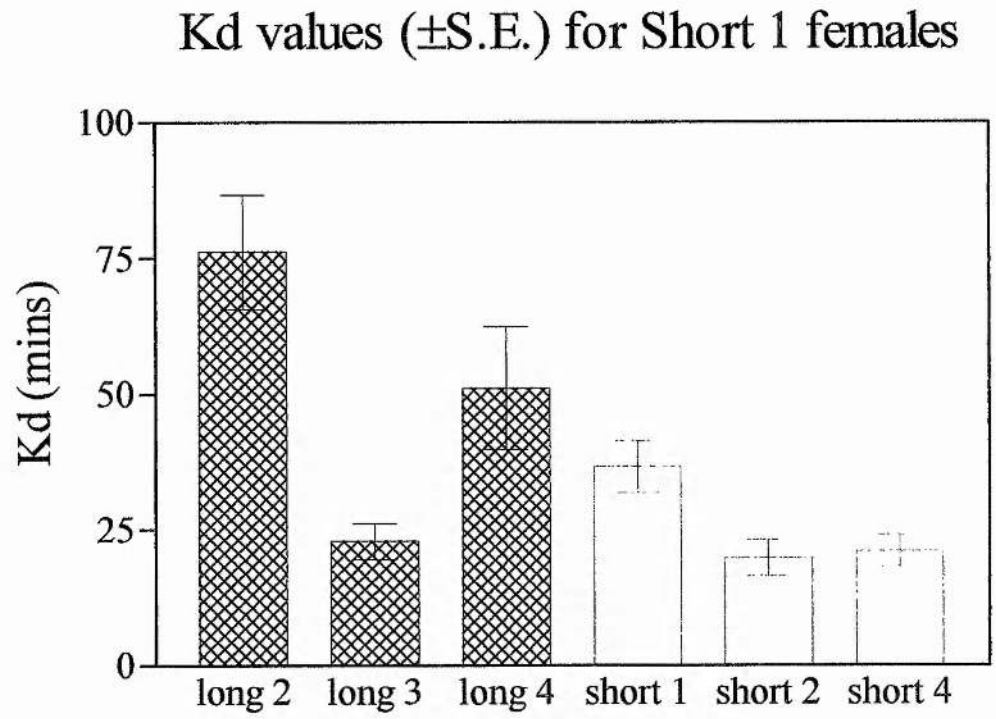


Figure 4.11 Kd values for Short 1 females to males of each replicate lines.

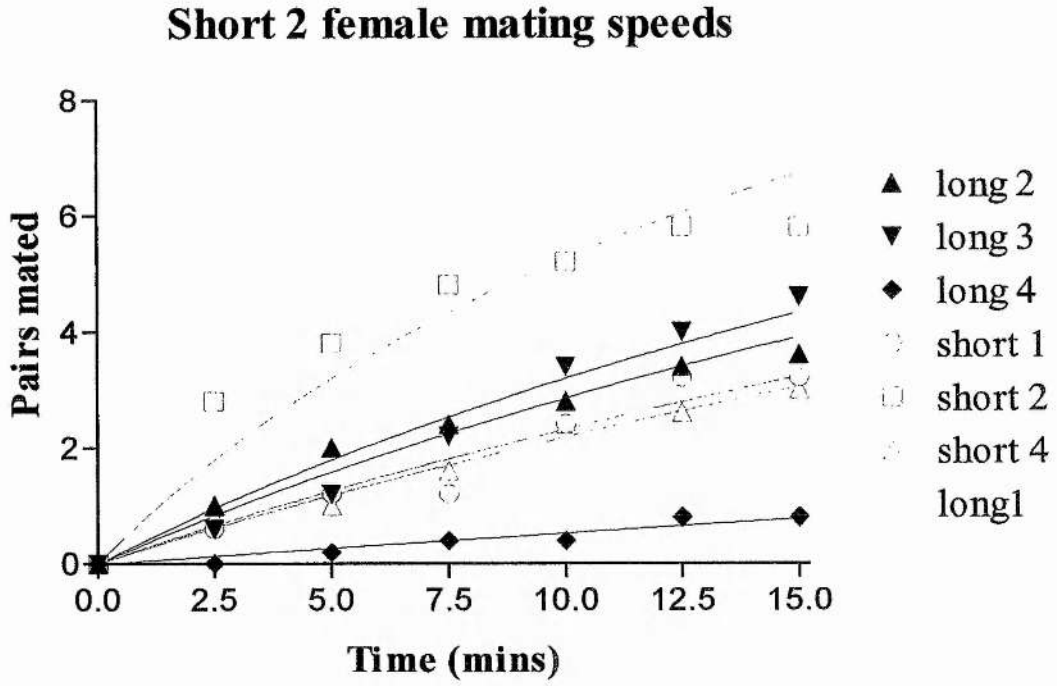


Figure 4.12 Average number of matings of Short 2 females with males from each of the replicate lines fitted with the non-linear regression.

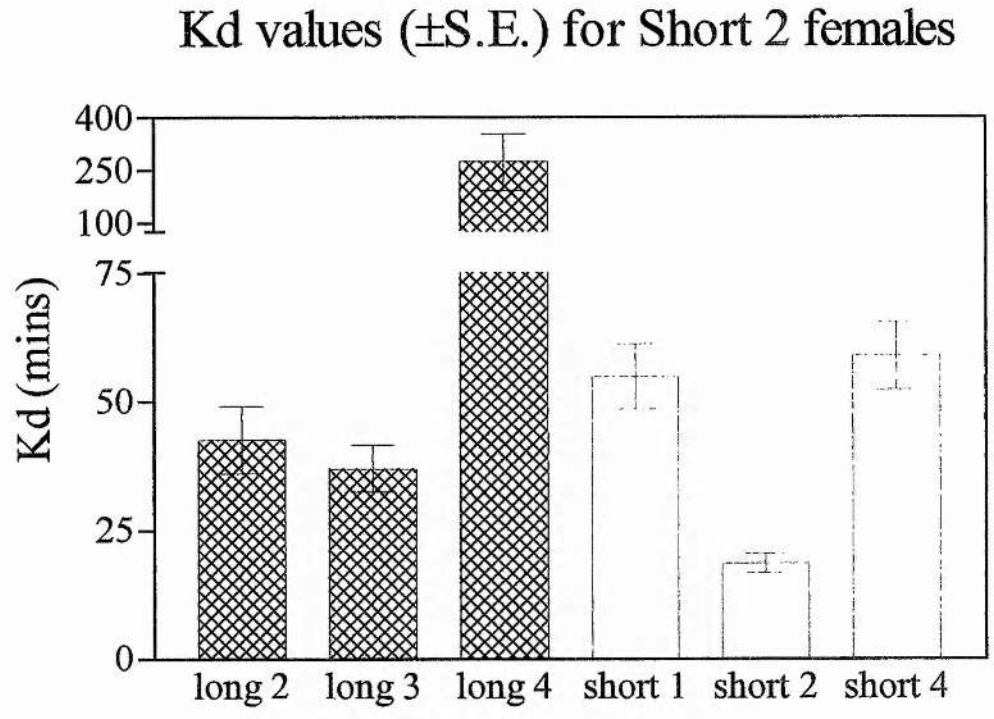


Figure 4.13 Kd values for Short 2 females to males of each replicate lines.

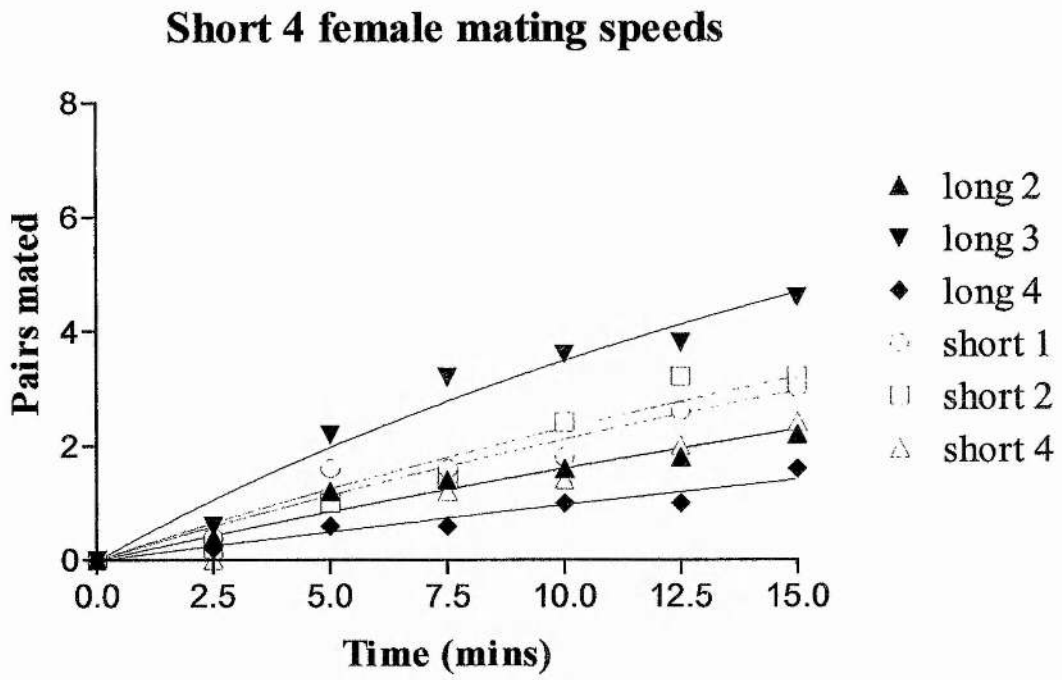


Figure 4.14 Average number of matings of Short 4 females with males from each of the replicate lines fitted with the non-linear regression.

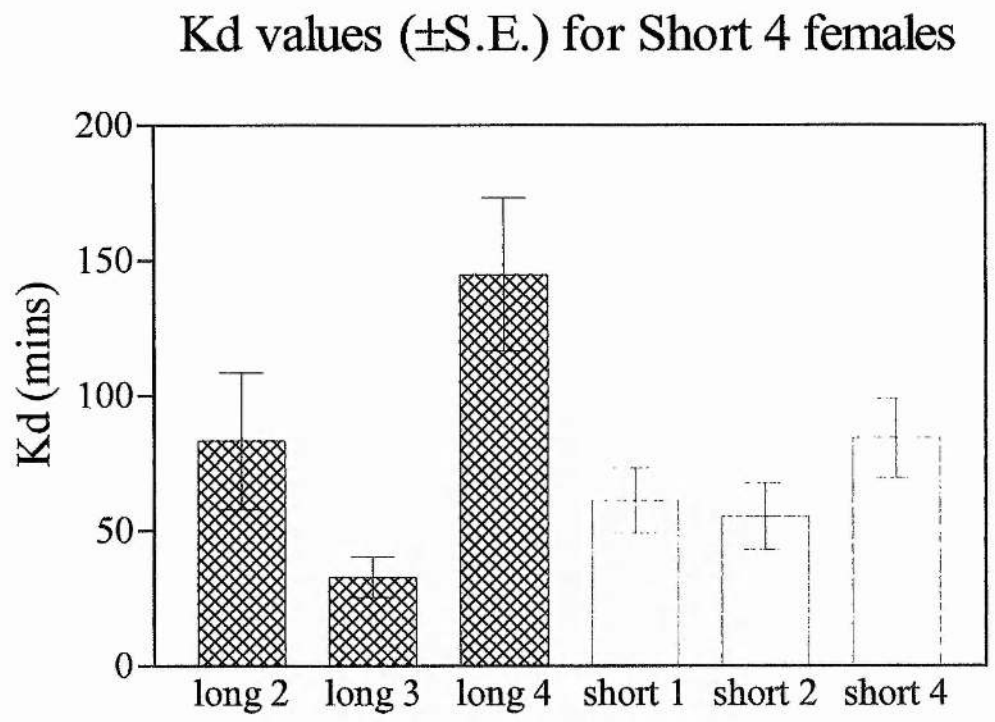


Figure 4.15 Kd values for Short 4 females to males of each replicate lines.

4.3.2 Female mating rate trials using winged and muted males.

Source of variance	DF	SS	MS	F	P
s/l female	1	6.446	6.446	1.14	0.291
s/l male	1	6.446	6.446	1.14	0.291
wing vs. wing-	1	375.446	375.446	66.26	0.000
s/l female * s/l male	1	2.161	2.161	0.38	0.540
s/l female * wing vs. wing-	1	3.018	3.018	0.53	0.469
s/l male * wing vs. wing-	1	17.161	17.161	3.03	0.088
s/l female * s/l male * wing vs. wing-	1	2.161	2.161	0.38	0.540
Error	48	272.000	5.667		
Total	55	684.839			

Table 4.4 Results of ANOVA of the number of pairs mating at fifteen minutes for the trials of short 2 and long 3 lines using winged and muted males.
s/l female = short 2 / long 3 females; s/l male = short 2/ long 3 males: wing vs. wing- = winged versus muted males.

Table 4.4 shows that there is no significant difference between short 2 and long 3 females, or between short 2 and long 3 males, nor was the interaction significant. There was a highly significant difference between trials involving winged males and muted males. However no significant difference was seen in the interaction between short 2 and long 3 males, with short 2 and long 3 females, when males are winged and muted. When the line of both male and female was compared with trials involving winged and muted males, no significant difference was seen. Therefore the only difference seen is a result of the amputation of the wings

Figures 4.16 and 4.17 show that over thirty minutes the difference in numbers mating between the wingless males of the two lines with females from the same lines do not diverge, and analysis shows that there was still no significant different between the four groups (table 4.5).

Source	DF	SS	MS	F	P
female	1	7.042	7.042	1.91	0.182
male	1	1.042	1.042	0.28	0.601
female * male	1	5.042	5.042	1.37	0.256
Error	20	73.833	3.692		
Total	23	86.958			

Table 4.5 Results of balanced ANOVA on the number of pairs mating at thirty minutes in trials involving muted males.

Figures 4.16 and 4.17 show the mating speed of Long 3 and Short 2 female lines respectively, with winged and wingless males from Long 3 and Short 2 over the course of the fifteen minutes of the trial. Figures 4.18 and 4.19 show the mating speed of Long 3 and Short 2 female lines respectively, with wingless males from Long 3 and Short 2 2 over the course of the trial period for the thirty minute wingless-only trials.

Due to the higher resolution between lines using the Kd value I examined the difference between trials involving winged and muted males separately. Analysis of the Kd values only showed a significant difference between trials of the different male lines using winged males ($F_{1,188} = 17.37$; $P < 0.0001$). For the trials involving muted males there was a significant effect of female line ($F_{1,300} = 6.18$; $P < 0.05$), and male line ($F_{1,300} = 13.12$; $P < 0.001$).

For the winged male trials, females mate significantly faster with Short 2 males (figure 4.20). The preference is also seen in the muted trials, as well as a higher rate of mating for short females (figure 4.21). In the muted male trials the preference for Short 2 males by females from both lines becomes significant. The differences seen are mainly due to the lower Kd value of Short 2 females with Short 2 males. Therefore preference is clearly not based on the variations in IPI.

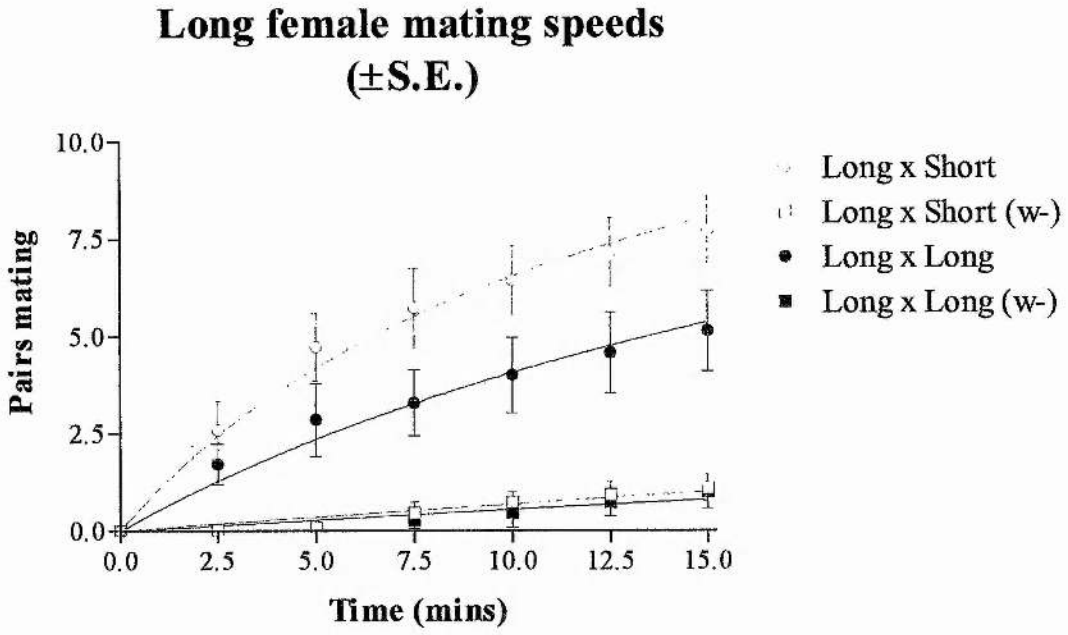


Figure 4.16 Numbers of Long 3 females with winged and wingless (w-) males mating over fifteen minutes.

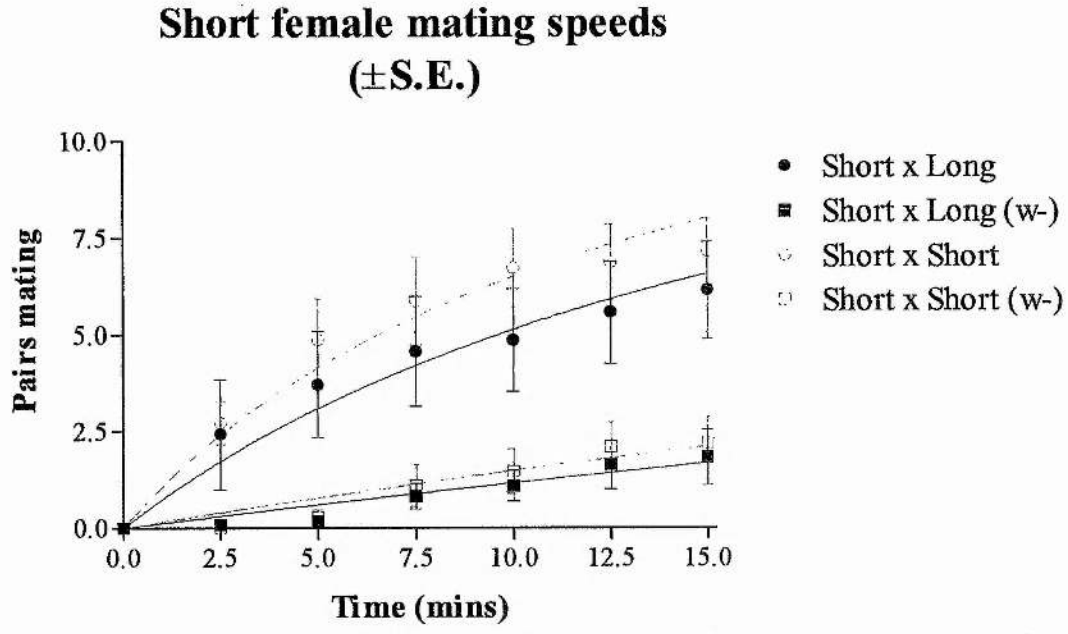


Figure 4.17 Numbers of Short 2 females with winged and wingless (w-) males mating over fifteen minutes.

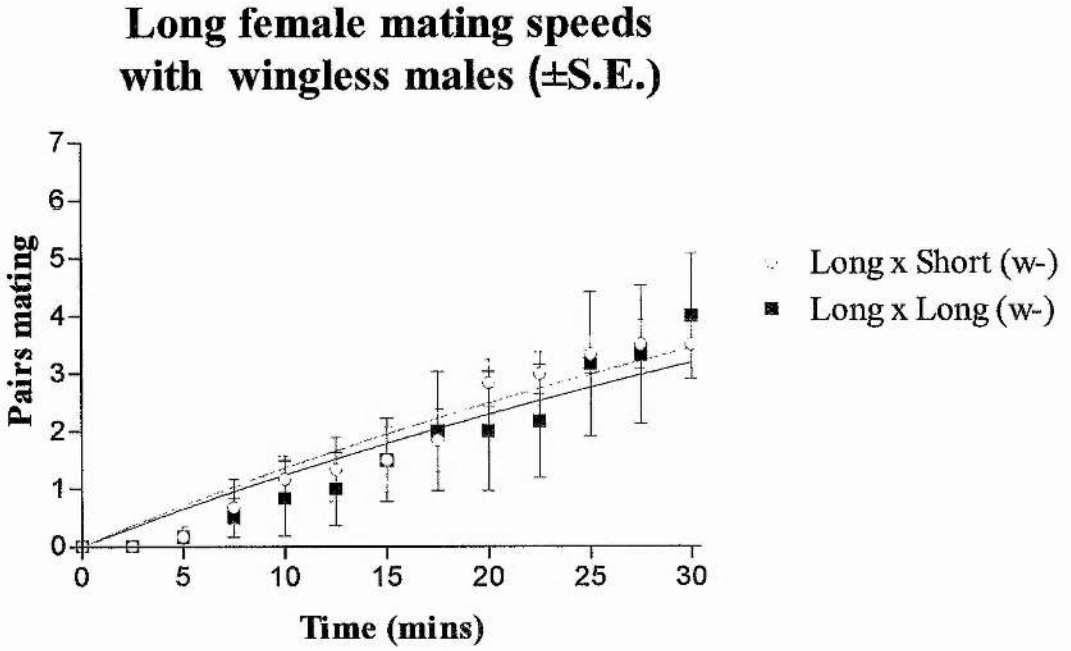


Figure 4.18 Long 3 female preference for Long 3 and Short 2 wingless males over thirty minutes.

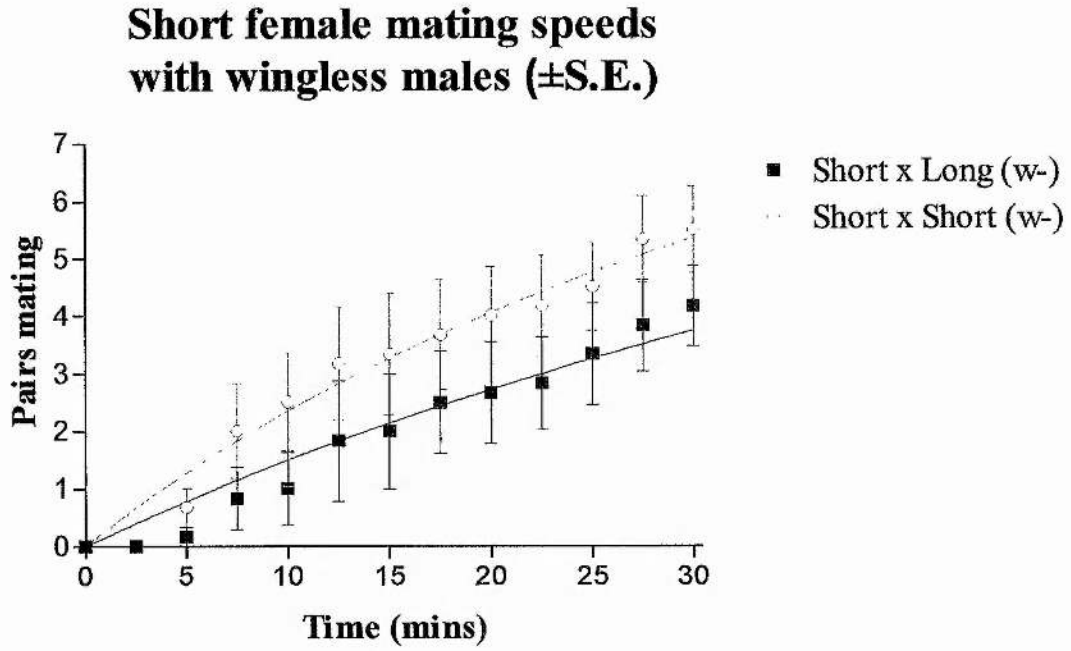


Figure 4.19 Short 2 female preference for wingless males over thirty minutes.

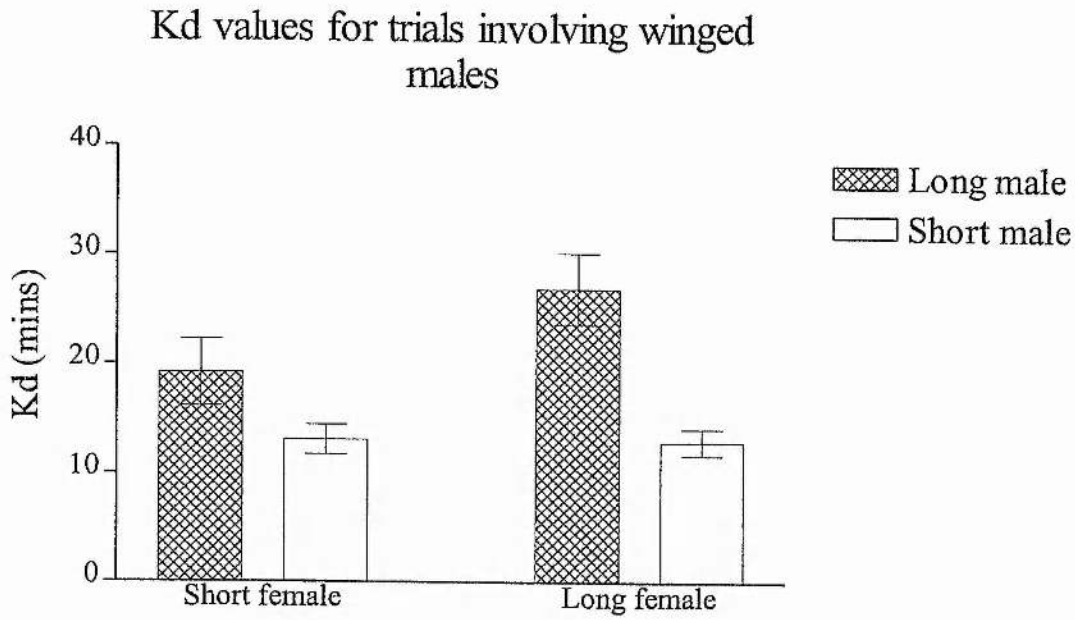


Figure 4.20 Kd values for winged male trials (with standard error bars).

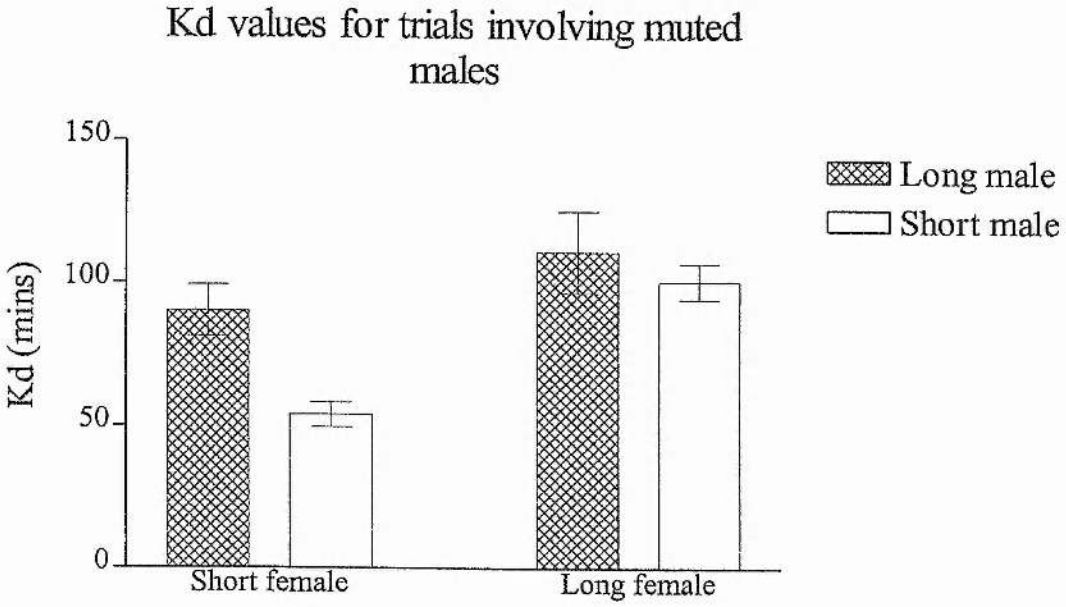


Figure 4.21 Kd values for muted male trials (with standard error bars).

4.3.3 Pietrastornina line female preference.

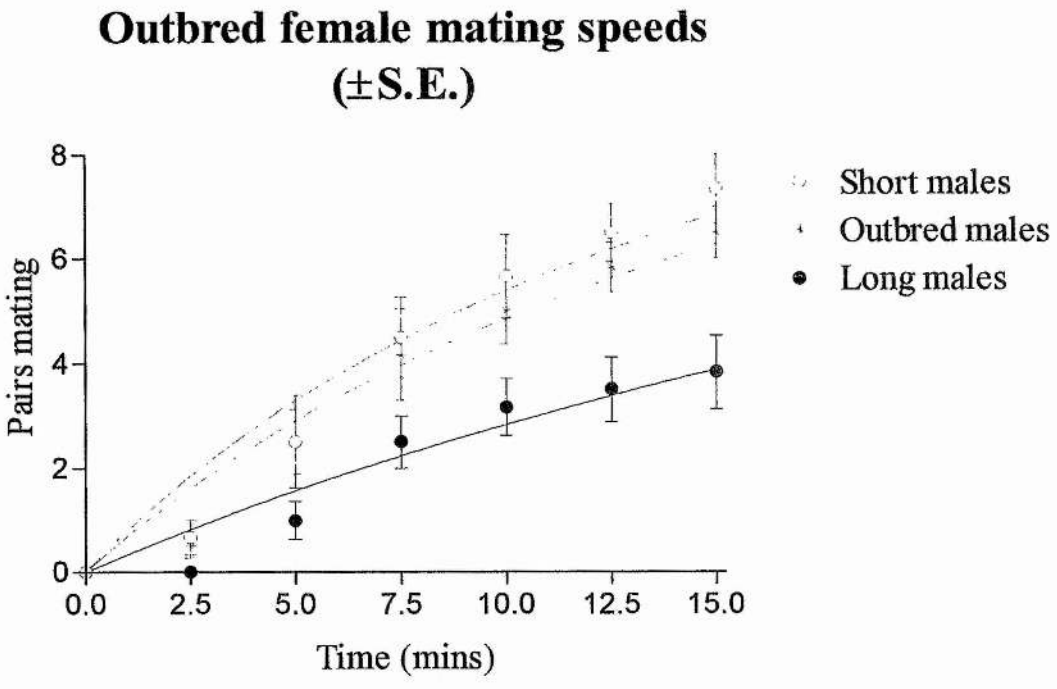


Figure 4.22 Pietrastornina line female mating speeds for Long, Pietrastornina, and Short line males.

The divergence seen in figure 4.22 between the mating levels of the three lines was significant from ten minutes ($F_{2,15} = 3.755$, $P < 0.05$ at 10 minutes; $F_{2,15} = 8.024$, $P < 0.005$ at 12½ minutes; and $F_{2,15} = 8.435$, $P < 0.005$ at 15 minutes). However, there was no significant difference seen in the Kd among the three lines. This is due to the large degree of variance in the Kd of the preference lines for the Long males. The average numbers of females mating with Short and Pietrastornina males is similar at all points measured. The preference for long males at each point measured was consistently the lowest value of males from each strain measured.

If the rates of mating seen in the no choice situation provide any guide to mating success, one would expect to see that males with a mean IPI value equal to, or less than the mean would normally achieve the highest number of matings. However, there is very little, if any advantage in having a shorter IPI. It would seem that it is

detrimental to have a long IPI, as these are discriminated against. If there is a cost to having short IPI over a long one, it would oppose the directional sexual selection against long IPI, if strong enough. Over time, stabilizing selection may have resulted in males with intermediate IPI, as is shown by Pietrastornina line males.

4.4 Discussion.

The objective of this experiment was to examine female mating speed with males who differed in IPI. The equation for a hyperbola used to model mating speed was used as the trials involved sampling, or mating of finite number of males with females, without replacement. Once a pair has coupled they do not have enough time to finish mating before the end of the trial and are permanently removed from the remaining pool available to mate, giving a maximum number of fifteen pairs mating. Subsequent availability of mated individuals after coupling would alter the position of the plateau, if indeed one would be reached.

In nature both sexes of *Drosophila* meet and mate on discrete patches of food such as rotting fruit (Speith, 1974). Time available for mating is limited as females will decamp if they do not wish to be mated. When there are two groups of males with a similar mating rate, but different plateaux which occur after the mating period, both will achieve similar levels of mating. The relevant factor is the initial rate, not the eventual position of the plateau.

The non-linear equation with a fixed plateau that was used was a better fit than the same equation with a variable plateau and a straight line. The rates of mating from equations with the same fixed plateau can be compared directly. Also the Kd value is derived from all data measured during the trial.

It is not known if all pairs would eventually mate given enough time. It has been shown that female *D. littoralis* discriminate against males that have had 30% or 60% of the wing length removed, when allowed a choice between these males and males with longer wing lengths. However, this is relative depending on the availability of other potential mates as males with wings that had 60% of the wing intact were discriminated against in choice experiments with fully winged males, but were favoured over males with 30% of wings (Hoikkala and Aspi, 1993). They found that in a no choice situation the number of pairs mating after fifteen minutes varied between groups of males with different wing lengths. Males with reduced wing length

that successfully courted females had to court for longer and display more bouts of song. This shows that in a no-choice situation a female will eventually accept a male in the absence of a more suitable choice. Females must balance desire to mate with the most suitable male, against the possibility that by being too choosy or rigid in her preference, and if her range of preference is too narrow, she will risk failing to obtain matings.

Tomaru *et al.* (1995) showed that female *D. biauraria* did not mate with heterospecific males of the closely related species *D. triauraria*, but did mate with *D. triauraria* males if the wings were removed to mute the song. Using wingless males and artificial song in a no choice experiment, heterospecific courtship song was shown to result in fewer matings than random noise and silence, thus inhibiting mating. It is unknown without further experimentation whether all females will mate with males regardless of how unattractive they initially appear.

The higher mating level and rate of mating by females with short males, regardless of the regime to which the female belongs, is seen across all three experiments. The higher mating success of short males is seen in the Pietrastornina line and has been maintained to some degree in the females from both selection regimes.

The female mating speed trial results show that males from certain lines mate at a faster rate with females from all lines. Another factor other than the IPI, that differs between male lines, such as the relative general vigour seems to be the principal factor. Subsequent song analysis of the lines showed that there was still a difference of 3.5 msecs between the long and the short lines.

In trials involving winged and muted males, the lines had initially shown an higher level of mating with males of their own song type. Short 2 females showed only a slightly higher number mating with Short 2 males over Long 3 males, as did Long 3 females, and assortative mating is unlikely to be occurring in either line. Removal of wings greatly reduced the levels of mating by the females, highlighting the importance of song in determining overall mating speed. However, the higher Kd

of Short 2 males was maintained, pointing to the cause of the difference being unrelated to IPI.

Although IPI does not seem to be having a major influence on mating speed in the selected females, it may still influence ancestral female preference. Pietrastornina females showed no difference in mating speed between Short and Pietrastornina males, suggesting that sexual selection for (or against) a shorter mean IPI is unlikely to be strong. However the difference in mating speed between the Pietrastornina line and the Long line is indicative of stronger discrimination. As the difference in IPI between the Pietrastornina and Long line is less than that between the Short and Pietrastornina line, female preference of the Pietrastornina line may be asymmetrical (figure 4.23). This agrees with the asymmetry of response to selection seen between the two selection regimes, indicating that the directional selection against longer IPI may have been effective in reducing heritability for IPI over a long period of evolutionary time. In evolutionary terms, males may have little to gain in terms of increased female matings by increasing the pulse rate, given the possible energetic cost, but much to lose by possessing a longer IPI. It may be that males with a longer IPI include some males whose longer IPI is due to lower viability.

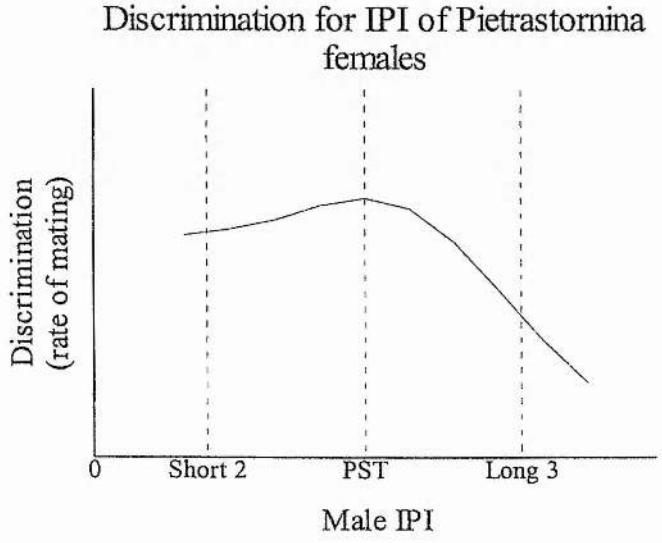


Figure 4.23 Discrimination curve of Pietrastornina females. (PST = Pietrastornina males)

Despite the change in IPI of the selection lines, there is no sign of assortative mating of females from long IPI lines with males with a long mean IPI over males with a shorter IPI. Any covariance of alleles for IPI and female preference is therefore weak or non-existent. There is still a hint of discrimination against long males, but this is much reduced compared to that seen in the Pietrastornina line, with no significant difference in numbers mating with long and short lines, and may also be masked by effects due to male fitness. Undoubtedly a factor other than mean IPI of males is more important to females, as can be illustrated by the fact that females from all but one line (Long 3) tended to prefer Short 2 males over males from the other short lines. If there is no association of fitness with IPI, females could not use this as a reliable indicator of fitness assuming a good genes model. However the convergence of the two selection regimes between the two rounds of selection may indicate that there may be such an association.

It may be that the maximum extent of the range of the female preference distribution curve has increased in the selection lines, or that the directional selection against longer IPI has been weakened or reduced (see figure 4.24). If females from which all replicate lines are descended showed the same discrimination as Pietrastornina females before the start of both rounds of artificial selection, this discrimination has been broadened. However the consistent trend of Short 2 females to mate with more wingless males from their own line over those from Long 3 demonstrate that there are factors which differ between lines beside mean IPI which affect females.

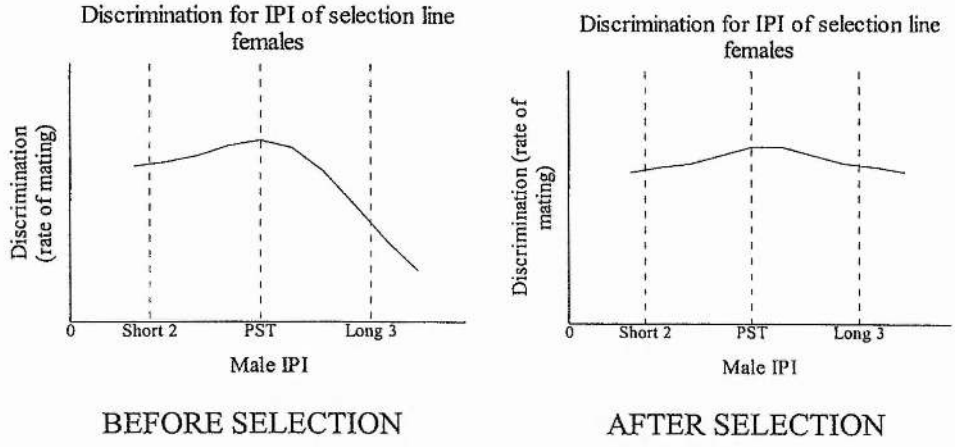


Figure 4.24 The possible change in discrimination by selection line females. (PST = Pietrastornina males)

Morris and Ryan (1996) studied the response of males and females of *Xiphophorus multilineatus* to the male signal (a pattern of vertical bars), which is absent in the sister species *X. nigrensis*. The bars act to deter rival *X. multilineatus* males, but *X. nigrensis* males do not respond to this male signal. However females from both species are more attracted to males with bars than without bars. This communication system consists of three components, the signal, the male response and the female response, with the signal and male response evolving more closely than the signal and female response. It is not known if the female preference is ancestral to the male trait (suggesting that this may be a case of sensory exploitation) or the trait predates female preference. What is clear is that the association between female preference and male trait is a weak one and as such unlikely to be closely linked at a genetic level. This study highlights the fact that although signal-response systems are usually thought of as 'dyads' of female preference and male choice, there are external forces acting on the system, which in this case is a 'triad' with male preference. These external factors may exert pressure on either the response or signal. The genes for these external factors may be more closely linked to genes for one half of the dyad than with the genes for the other half. Ryan and Rand (1993) show how

female preference may be divergent from the male trait. The difference in female preference and the calls of conspecific male Tungara frogs illustrate how trait and optimal preference can be different.

During artificial selection, mating was arbitrary. It is therefore a possibility that the covariance of male IPI and female preference have diverged, similar to that seen for preference for skin pigmentation in guppies during selection by Breden and Hornaday (1994). This would result in the loss, or dilution of female preference for males with a relatively short IPI as seen in the outbred Pietrastornina females. Populations from the wild which already show differences in IPI should be studied to confirm if this is the case.

CHAPTER 5: SONG AND FEMALE MATING SPEED OF THE ZIMBABWE STRAIN OF *DROSOPHILA MELANOGASTER*

5.1 Introduction

Speciation involves the divergence of two populations that do not initially have pre- or post-mating barriers, resulting in distinct species with barriers to reproduction. At this stage the two populations are thought of as distinct species as per Mayr's definition of species (1942). The process itself is likely to be gradual, with the two populations acquiring changes in behaviour and morphology involved in speciation. Coyne and Orr (1989) showed that the accumulation of premating isolation between different species increased at a steady rate with increasing divergence between species.

One is required to study the process at one point along a continuum. One approach is to study isolation in two species that have recently diverged, assuming that the present isolation between the species has come about recently. Examples of studies of isolation in two recently diverged species include examinations of the differences in song within the *Drosophila biaurauria* complex (Tomaru and Oguma, 1994a); genital morphology (Coyne, 1983) and pheromones within the *D. melanogaster* complex (Coyne *et al.*, 1994).

A different approach is to select for differences within a population to examine the nature that the change in a trait might take, and the genetic nature of the change. Methods to study this include the use of artificial selection to create differences between two or more groups derived from the same initial population. Selection can then be followed by examining the genetic nature of the change in trait and also any change in female preference for the trait. (i.e. Ritchie and Kyriacou, 1996).

Studies of isolation in populations displaying differing levels of pheromones or other behavioural traits known to produce premating isolation within a species are of great interest. The changes have not been artificially selected for and are the result

of sexual selection or natural selection due to differing environmental conditions. This is the study of possible speciation in progress, as opposed to the potential for isolation and speciation, i.e. the artificial selection experiment of chapter 3, or the isolation existing after speciation examined in chapter 2.

The range of *D. melanogaster* has expanded from sub-Saharan Africa to Europe and the rest of the World since the last glaciation (David and Capy, 1988). Some studies of premating isolation among different geographic strains have not revealed premating isolation ({29 strains} Henderson and Lambert, 1982; {4 strains} Van den Berg *et al.*, 1984). In contrast, studies of matings with single pairs over less than 24 hours have shown differences in mating speeds between strains (Connolly *et al.*, 1974; Eastwood and Burnet, 1977). Using mass mating techniques, Cohet and David (1980) found slight indications of sexual preferences of females for their own males to males from other strains, but did not find reproductive isolation between a French and African strain.

Gene flow within and between populations from different continents is extensive, and the degree of genetic variation is mostly within rather than between populations (Singh and Rhomberg, 1987). However Begun and Aquadro (1993) showed that a Zimbabwe strain of *Drosophila melanogaster* was twice as variable as USA strains at the DNA sequence level. Also there were nearly fixed differences between the two geographic locations for genomic regions of low recombination. These results demonstrate a degree of population structure not indicated by previous studies within *Drosophila melanogaster*. The greater amount of variation in the African populations suggests that there have been a series of bottlenecks during global expansion. The maintenance of the genetic diversity of the African populations is either by specific selection pressures on them, or assortative mating between different populations of *D. melanogaster*. It does not support the idea of an effective global population with free gene flow, which would decrease the variation both within and between local populations.

Wu *et al.* (1995), examined the mating preferences of the different populations studied by Begun and Aquadro for any indication of premating isolation. They found variation in female preference for males in no-choice and mate choice experiments, with a significant degree of premating isolation shown between the Zimbabwe females and males from other strains. Also females from the non-African strains showed reduced preference for the Zimbabwe males over those of non-African strains. Although the preference was not as strong as for the Zimbabwe females, it was still statistically significant. The level of discrimination was not as large as between *D. melanogaster* and its sibling species *D. simulans*. Work by H. Hollocher (personal communication) has not found a major difference in the pheromonal composition between the Zimbabwe strain and other *D. melanogaster* strains that could account for the significant mating isolation between the strains. Therefore the cause of the premating isolation is not clear.

Difference in mean IPI between species has been shown to be important in sexual reproduction between species in the *D. melanogaster* complex, and may play a role in the isolation of the Zimbabwe strain. I wished to measure the courtship song to determine if mean IPI of male courtship song varied between the Zimbabwe strain and the non-African control strains.

Mating speed trials were used to assess likely preferences of Zimbabwe and non-African *D. melanogaster* females. The Short 4 selection line (chapter 3) was also used because the mean IPI is similar to that of the Zimbabwe males.

5.2 Methods.

5.2.1 Stocks.

All Zimbabwe stocks [Z(S)30, Z(H)28, Z(H)42 and Z(S)53] are isofemale lines from the Sengwa Wildlife Reserve, Zimbabwe (Begun and Aquadro, 1993). The strains used are from the same population as those used by Wu *et al.* (1995).They were not used in that experiment but show stronger pre-mating isolation than those used (H. Hollocher pers. comm.). The two control stocks were from High Grove, California (HGCA) and from France (FR). All stocks used were obtained from Dr. Hope Hollocher, Princeton University.

The Short 4 was derived from a wild caught stock from Pietrastornina, Italy. The Short 4 line resulted from the artificial selection for short mean IPI (see chapter 3), and had shown the greatest response to selection, with a mean IPI value of 29.19 msecs, similar to that of Z(H)42. The Short 4 line was assumed to have effectively the same mean IPI as Z(H)42.

Each stock was maintained in two replicate 30 ml vials containing standard medium (chapter 2). All vials were stored in a fan cooled incubator on a 12/12 hour light/dark cycle set at 24°C. Each generation was established by mass transfer of about eight to twelve adults to new vials containing fresh media, then the flies were removed after three days. Exact numbers of adults were not measured, and larval density was not directly controlled.

5.2.2. Song analysis of Zimbabwe stocks.

Single males were isolated within twenty-four hours of eclosion and placed into 30 ml glass vials containing medium. Two day old males were allowed to equilibrate to ambient temperature before aspiration into the courtship chamber, without anaesthetic. The chamber contained a virgin female of the France control line, collected within twenty-four hours of eclosion, and muted by amputation of the wings under CO_2 anaesthetic on the day of recording. Recording started after the male produced one burst of pulse song, and continued for the next five minutes of courtship. All recordings were carried out during the twelve hour light period of the diurnal cycle. All lines were recorded in every session and sequentially interleaved. Fluctuations in temperature were limited using external heaters and fans and held close to 24°C.

The songs were recorded onto analogue cassette tape using a Marantz cassette recorder set at a fixed recording level. The recording was filtered (high pass 250 Hz, low pass 1 KHz), and digitized by a 1401 analogue-digital converter (sample rate of 4 MHz), then written as a SPIKE2 (C.E.D.) formatted file on an IBM compatible computer. The files were analysed using a SPIKE2 software prewritten analysis program FASTDMEL.TXT, and all subsequent manual editing of events was done using EVENT.TXT (for description see chapter 2).

The unadjusted mean IPI's of flies were grouped by stock, and regressed against temperature. The regression coefficients of the different strains were not significantly different from each other, and all IPI values were adjusted to the values expected at 25°C for temperature using the regression coefficient of all individuals tested. Variation in mean IPI between the different lines using the adjusted mean IPI values was tested for significant difference by one way ANOVA.

5.2.3. Female mating speed trials.

For each stock, individuals were collected upon eclosion, anaesthetised by CO_2, sexed and then separated by sex. Adults were stored in 30ml vials containing standard medium, with between ten and twenty individuals per vial, for two to three days.

In each trial all flies were of the same age, preferably two days old. For each trial fifteen flies of each sex were anaesthetised and placed into a fly mating chamber as described in chapter 4. The flies were allowed one minute to settle and recover before removal of the barrier started the trial. The number of pairs copulating was noted at two and a half minute intervals for the following twenty minutes. Seven replicates of nine groups, which included combinations of all three lines were done. All stocks were recorded in the same session to minimise variation caused by differences in conditions between sessions.

All flies were used for one trial unless no matings occurred during the entire trial. Individuals from such trials were anaesthetised again, separated by sex and reused after at least one intervening trial. No flies were used for more than two trials. Males were not retested with the same females from the first trial, or with females from same line as those females.

All trials were mixed randomly by combination, and were performed during the twelve hour light period. The trials were carried out over two successive generations of each stock. Equal numbers of trials for each combination were performed per generation. Flies from Short 4 were from generations twenty-six and twenty-seven after the selection experiment.

5.2.4. Data analysis of trials

The Short 4 line was used as a "positive control" to test the response of Z(H)42 females to males from a different line, but with a similar mean IPI to males from their own line. The France line was used as a control line, representing a European continental strain.

To examine any differences between the average numbers of females of the different lines mating with males from the different lines the data were analysed by the General linear model in MINITAB. Numbers mating at ten and twenty minutes after the start of the trials were tested for significant differences. The data were also square root transformed due to the large number of small values and zero scores. The transformed data were analysed in the same manner.

I also examined the relative rates of mating of each combination by comparing Kd values from a non-linear regression with a fixed plateau of 15 pairs. The regression equation is a hyperbola, described in chapter 4. The equation for the curve is $Y = 15*X/(Kd + X)$. Kd is the time at which half maximal binding is reached, and is inversely proportional to the rate of mating. The choice of the hyperbola curve for mating speed trial data is discussed in chapter 4.

5.3 Results

5.3.1 Song recordings

The temperature range of all recordings was 23.65 - 26.4°C (mean 25.4°C, S.D. = 0.5°C). The slopes of the linear regressions of temperature to IPI did not significantly vary from the original regression value of the Pietrastorina stock used by Ritchie and Kyriacou (1996) and for the Short 4 selection stock (chapter 3). The mean values for each stock using own line adjusted values, and France adjusted values were not significantly different from each other using two-sample t-tests. Unadjusted IPI, and IPI adjusted for temperature using both own line and France regression values are shown in table 5.1. The mean IPI of Short 4 was 29.98 ± 0.43 S.E. msecs (chapter 3).

Stock	N	Unadjusted IPI (±S.E.)	Adjusted IPI (±S.E.)
Z (H) 42	21	29.50 (0.35)	28.97 (0.48)
Z (H) 28	11	30.62 (0.98)	29.91 (0.95)
Z (S) 30	14	32.18 (0.67)	31.62 (0.70)
France	35	31.98 (0.26)	31.43 (0.35)
HGCA	15	34.27 (0.28)	33.93 (0.30)

Table 5.1 Mean IPI values of all Zimbabwe and control lines.

There was a significant difference in mean IPI among all lines ($F_{4,95}$=12.18, P<0.0001), and also of the three Zimbabwe $F_{2,43}$=4.47, P<0.05) using one-way ANOVA.

Posthoc testing showed that the mean IPI of Z(H)42 is significantly lower than all other groups except Z(H)28. The mean IPI of HGCA is significantly higher than

all other groups. Therefore the IPI of the France line was significantly different from both the HGCA and Z(H)42. As the France control sung more readily and had had more individuals measured I chose to examine the difference in mating speed between the France and Z(H)42 stocks. An examination of HGCA with the Zimbabwe stock may also have been used. The difference between the latter pair is greater. However onely one line was used.

5.3.2. Female mating speed trials.

Male↓\Female→	Short 4	France	Z (H) 42
Short 4	4.5714	4.2857	1.7143
France	4.4286	6.4286	0.1429
Z (H) 42	3.8571	6.1429	1.1429

Table 5.2 Mean number of pairs mating by combination at the end of the trial.

The mean number mating by the end of the trial, for each combination is shown in table 5.2. There was a significant difference in numbers mating among the three female lines due to females from the Z(H)42 line showing a much lower rate of mating from all lines than females of the other two stocks. The results from the statistical analyses are shown in Table 5.3. There was no significant difference in the number of males from different lines mating with females of each line. There was also no significant male-female interaction.

Time	Original Data	Transformed Data
10 minutes	$F_{2,54}$=16.73, P<0.0001	$F_{2,54}$=24.43, P<0.0001
20minutes	$F_{2,54}$=21.99, P<0.0001	$F_{2,54}$=29.57, P<0.0001

Table 5.3 Analysis of mating levels using GLM showing the level of difference of females from all lines with males of the different lines.

The numbers of Z(H)42 females mating with males from each line showed a consistent relative order from five minutes after the start of the trial period (Figure 5.1). Short 4 males obtained most mating, and France males the least. The comparison of Kd values illustrated in figure 5.2 shows that there was a significant difference among the three male lines ($F_{2,183}$=18.61, P<0.0001) using one-way ANOVA. It can be seen that although mating speed with Z(H)42 males was not significantly different from Short 4 males, both of these lines were significantly different from that of France males. This is consistent with the hypothesis that the Zimbabwe females discriminate on the basis of mean IPI. Females prefer a lower mean IPI, such as that displayed by males of their own line and the Short 4 line. The results for the Z(H)42 females agree with the results of Wu *et al.* (1995), but the generally low levels of matings by Z(H)42 females means that Z(H)42 males mate more readily with France females.

Females of the Short 4 line showed no significant difference in mating speed for males from different stocks (Figure 5.3). The Kd values were also not significantly different (Figure 5.4). This result concurs with the findings of the female mating speed trials or the selection lines in chapter 4, which showed no difference in mating speeds of short line females with males of different lines on the basis of IPI.

France females showed a consistent relative order of matings with males from each line of the three lines tested at all points measured (Figure 5.5). France males obtained most matings, followed by Z(H)42 males, with Short 2 males obtaining least.

The Kd values shown in figure 5.6 were significantly different ($F_{2,183}$=19.54, P<0.0001). This result was due to the difference between both France and Z(H)42 males compared with Short 4 males. France females do not show a difference in mating speed between their own males and Z(H)42 males.

The lower mating speed with Short males cannot be due to the difference in IPI, given that Short and Z(H)42 males have an effectively equal IPI. Therefore some other unmeasured factor would have to be responsible. The difference in female mating speed with Short and Z(H)42 males is not seen with Short and Z(H) 42 females.

Figure 5.1 illustrates the differences in mating speed of Z(H)42 females to males from the three lines, while figure 5.2 shows the differences in Kd, or rate of mating for the same results. Figures 5.3 to 5.6 show the results in the same manner for France and Short 4 females.

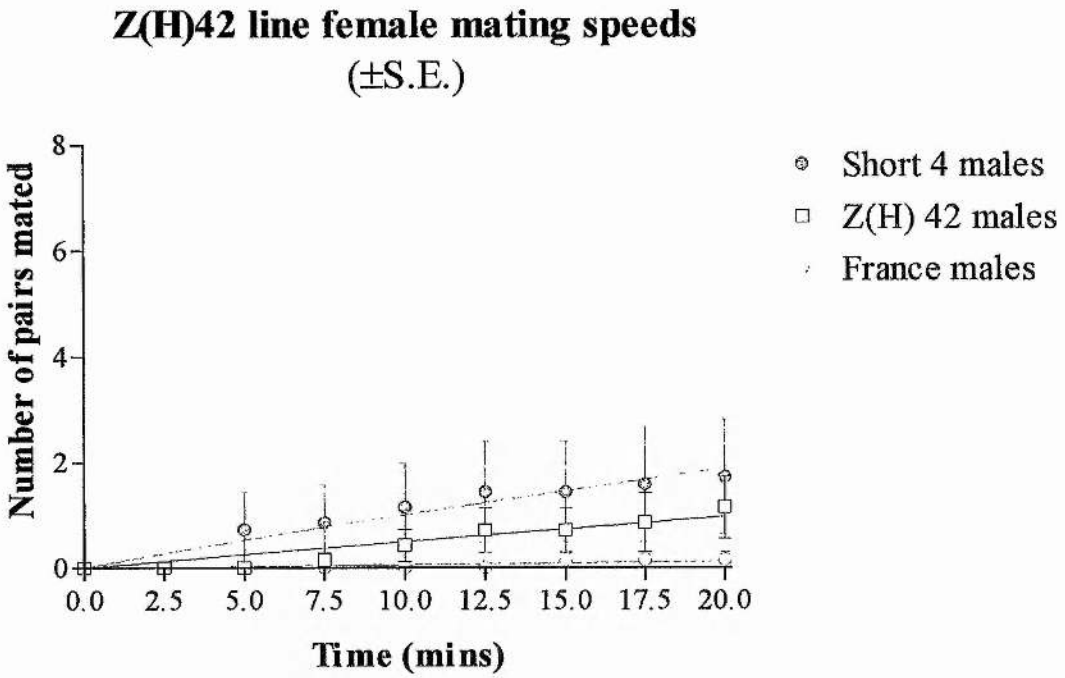


Figure 5.1 Levels of matings of Z(H) 42 females with males fitted to the non-linear regression curve [Y = 15 * X/(Kd + X)] of Short males (red); Z (H) 42 males (black); and France males (blue).

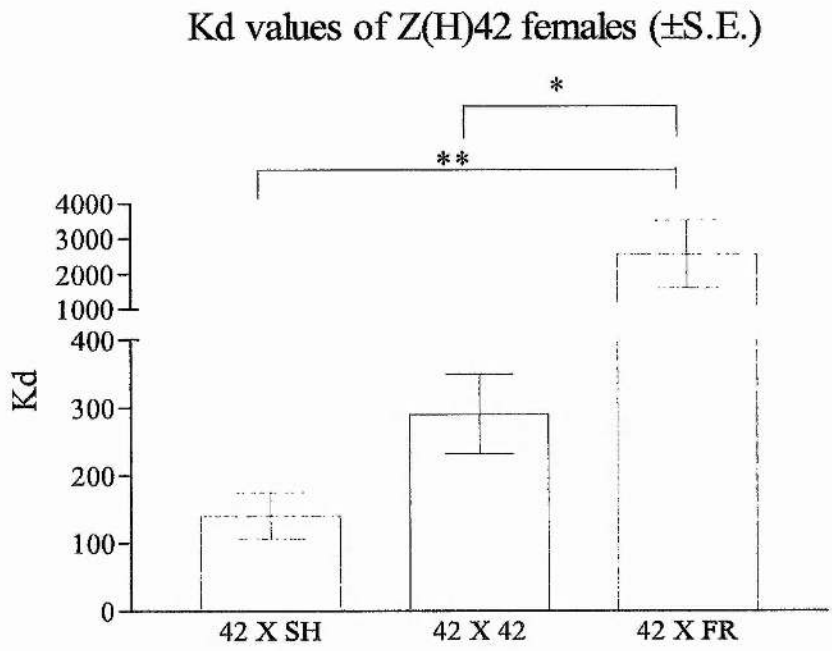


Figure 5.2 Kd values of the Z(H)42 females for males from the three lines (Short 4 = 42 X SH; Z (H) 42 males = 42 X 42; and France males = 42 X FR), with significant differences shown.

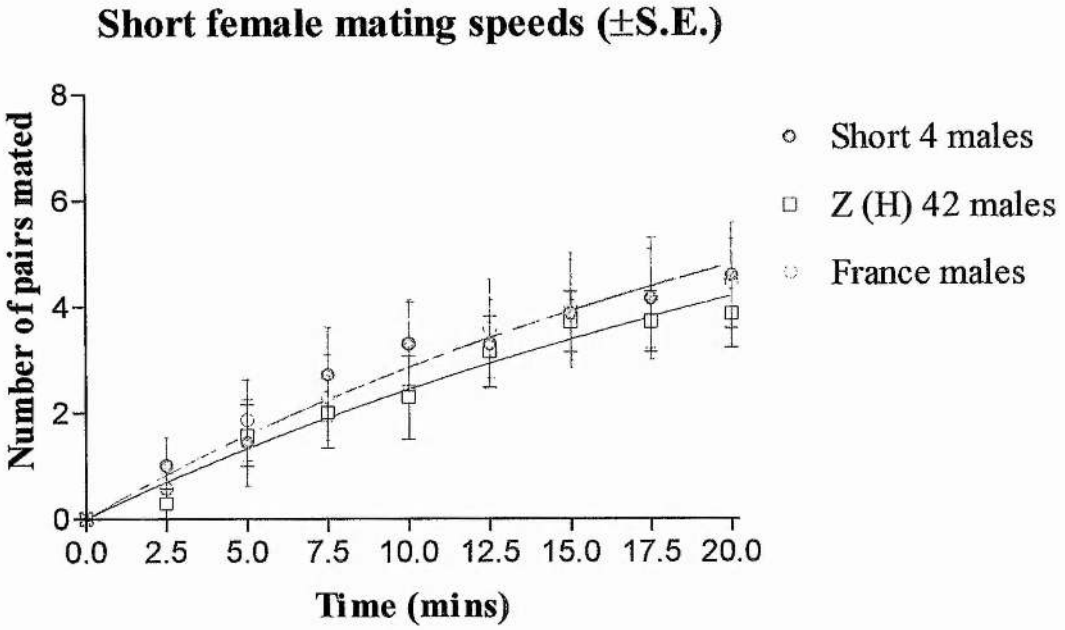


Figure 5.3 Levels of matings of Short 4 females with males fitted to the non-linear regression [Y = 15 * X/(Kd + X)] of Short males (red); Z (H) 42 males (black); and France males (blue).

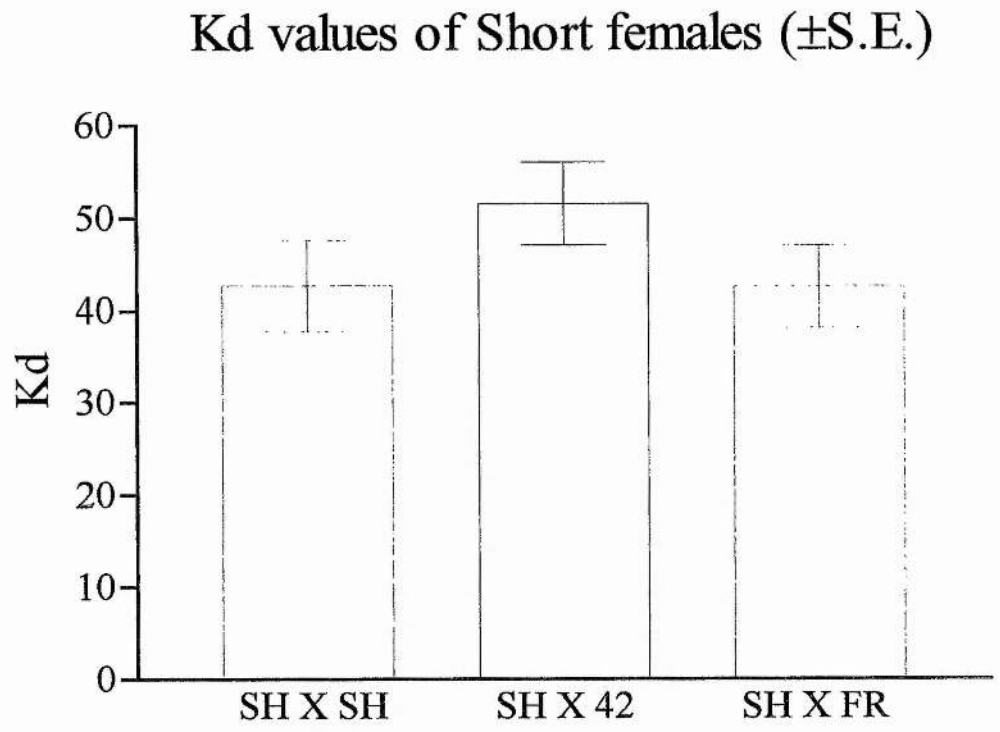


Figure 5.4 Kd values of the Short 4 females for males from the three lines (Short 4 = SH X SH; Z (H) 42 males = SH X 42; and France males = SH X FR).

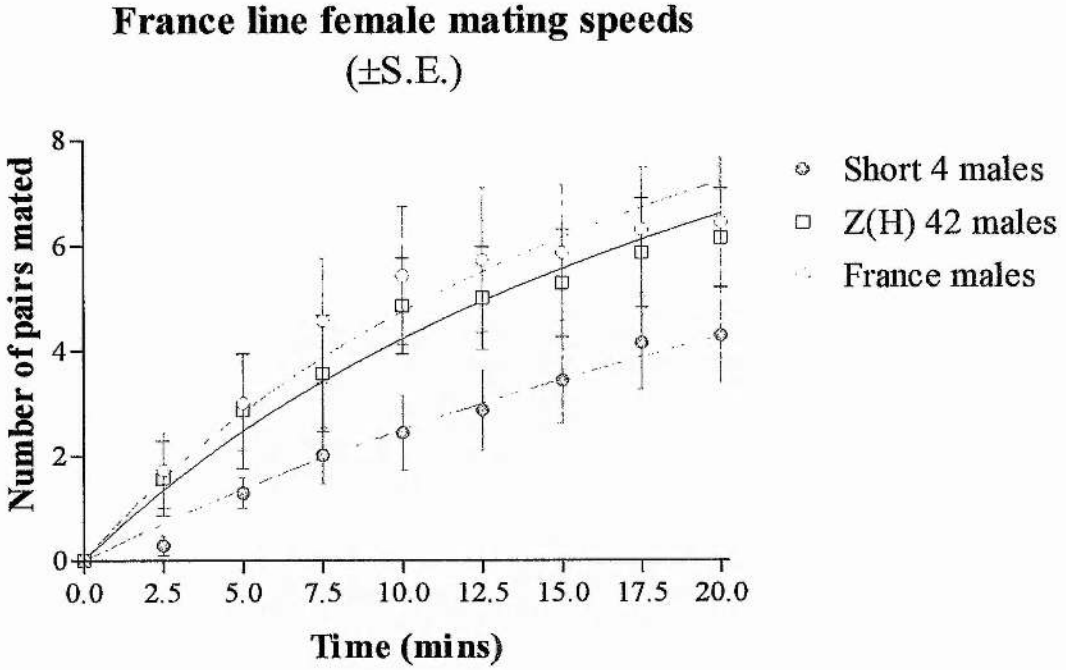


Figure 5.5 Levels of matings of France females with males fitted to the non-linear regression curve [Y = 15 * X/(Kd + X)] of Short males (red); Z (H) 42 males (black); and France males(blue).

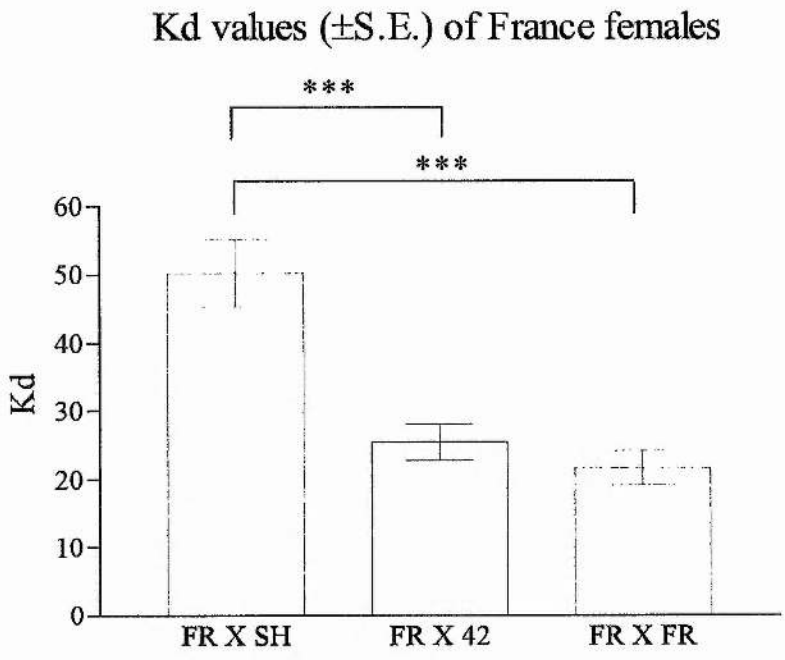


Figure 5.6 Kd values of the France females for males from the three lines (Short 4 = 42 X SH; Z (H) 42 males = 42 X 42; and France males = 42 X FR), with significant differences shown.

5.4 Discussion.

The three Zimbabwe strains have lower, or equal mean IPI compared to the two control stocks. This shows a difference in IPI between populations that could contribute to the sexual isolation seen by Wu *et al.* (1996). This highlights the active role IPI may play in speciation.

Due to the relatively low level of mating by Zimbabwe females, one would expect males to mate with females from a different population that showed a greater readiness to mate. The result would be a genetic mixing of the Zimbabwe population with other populations through these male mating.

The reason for the low level of mating by Zimbabwe females may be a consequence of an adaptation to conditions of their native environment. One possibility may be temperature, with the females mating at a higher rate at higher temperatures. If this was the case then mixing would be prevented.

Wu *et al.* (1995) had problems with lower levels of mating in no choice experiments, which seems to have been the reason for the change to mate-choice trials. They found discrimination by Zimbabwe females for own males over control males in both no choice and choice trials. It would therefore seem reasonable to expect that the discrimination seen by females in this study would have also been reflected in choice trials.

The similarity of male mating success suggests that the premating isolation of the Zimbabwe strain is due to female preference for the mean IPI of Zimbabwe males over the higher France IPI. Given that the Z(H)42 female mating speed with Short males was not significantly different from Z (H) 42 males, mean IPI would explain the discrimination against France males. The lower mating speed of France females with Short males suggests that *D. melanogaster* females are not selecting for males with the shortest IPI as has previously been suggested (Ritchie and Kyriacou, 1996). The similarity between France female mating speeds with France and Zimbabwe males does not reflect the results of Wu *et al.* (1995). The disparity of results may be

due to the use of a no-choice method. The choice method favoured by Wu *et al.* (1995) may have given a higher resolution to differences between mating speeds of France females with France and Zimbabwe lines.

What is somewhat suprising is that the mean IPI of the HGCA line, which had been used as a control line, showed a significantly higher value than the France stock. As the mean IPI of HGCA is significantly different from France, HGCA could not be used as a control alongside France. The selection of the France line as the control line was for practical reasons. However due to the higher IPI which is closer to the values reported by Ritchie *et al.* (1994) it may have been better to use HGCA as the control. It is impossible to say if the value of the France line is low compared to an average species mean IPI or whether the HGCA mean is high from this experiment, as there can be variation between recording sessions even for the same population. If females are discriminating against the France males on the basis of IPI then the discrimination would be greater with HGCA males. Repeating the experiment with HGCA lines and other geographic strains would be of further interest.

There is greater variation in mean IPI of these geographic populations than that of European populations, which are highly stereotypical (Ritchie and Kyriacou, 1994). If gene flow between populations from different continents is extensive, the high stereotypy seen in European strains should be reflected world-wide. It also calls into question the assigning of an average global mean IPI for all populations of *D. melanogaster*.

All Zimbabwe stocks represent isofemale lines from the same population. Therefore the difference seen between Z(H)42 and the other two Zimbabwe strains would suggest large variation in mean IPI within the population. The mean IPI of Z(H)42 may represent the IPI of males at the lower end of the original population's distribution. The mean IPI of the ancestral strain may be about 29 msecs. This would give a difference of 2 msecs below France and 4 msecs below HGCA. This difference is comparable to that achieved after strong artificial selection for IPI (Ritchie and Kyriacou, 1996; Chapter 3). Ritchie and Kyriacou (1994) suggested that isofemale

lines may increase the variability of IPI compared to that seen in the original population due to epistatic variation being converted to additive variation. It is possible that the difference in mean IPI between the three Zimbabwe lines is an exaggeration of the variation seen in the original wild population.

A comparative study involving more geographic strains would clarify the relationship of the populations measured to a species mean IPI, and measure the range and global variation in mean IPI within the species. A high degree of population structure within the species has been proposed by Begun and Aquadro (1993) and Ferveur *et al.* (1996). A highly structured variation in mean IPI between African strains and other continental strains agrees with this hypothesis.

The mean IPIs of Z(H)42 and Short 4 stocks have not been recorded in the same session. It may be that there is a difference in IPI between Short 2 and Z(H)42 with the former having a slightly lower IPI. If it were the only cause for the higher mating speed of Z(H)42 females for Z(H)42 males over France males, the range of the Z(H)42 female discrimination curve would be narrower than that of France females. Discrimination might also occur over a different range with the fall off in discrimination between the IPI seen in Z(H)42 males and France males. There would have to be a sharp fall off at the end of the preference curve with a broad plateau as shown in figure 5.7. This would be the only way to explain the similar mating speeds with Z(H)42 and France males. The distribution curve represented here is similar to the hypothetical preference function *a* in the model by Ryan and Rand (1993) (see introduction). Such a curve would mean that preference for a trait could vary widely with the preference range, but with distinct boundaries to that preference.

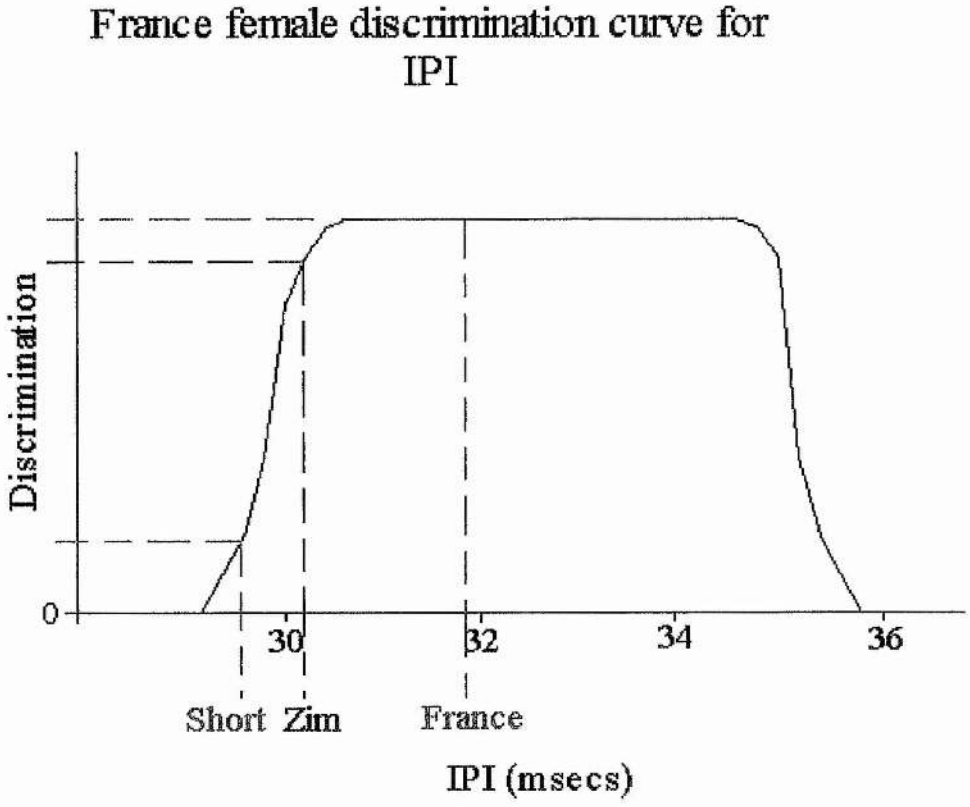


Figure 5.7 Hypothetical France female discrimination (relative mating speed) for IPI showing the response to males from the Short (red), Zimbabwe (black) and France (blue) lines

Ferveur *et al.* (1996) studied levels of 7,11 heptacosadiene (7,11 HD), which is associated with induction of male wing vibration, in 63 geographic strains. He found that strains from Sub-Saharan Africa and the Caribbean both had low levels of 7,11 HD, while the other strains had high levels. Furthermore, females from strains with high levels of 7,11 HD showed higher levels of matings with both morphs of male. This was due to increased levels of sexual activity in the males from both the high and low 7,11 HD morphs. However no discrimination by females between males of the two morphs was seen. The spread of *D. melanogaster* to the Caribbean is thought to be direct from Africa due to the slave trade. The high 7,11 HD morph is therefore thought to be the derived state.

African strains of *D. melanogaster* are thought of as ancestral to European and American strains. The lack of preference by France females compared to Z(H)42 females for males with a different mean IPI, or its broader range, may be due to the former being from a 'derived' population. Females of a derived population might lack the discrimination for a narrow range of mean IPI. Males with a wider range of IPI would obtain matings.

The discrimination shown by Z(H)42 females is discrimination against the 'derived' males by the females of the ancestral stock that was not shown by females from the derived populations. This therefore does agree with Kaneshiro's hypothesis (1976). The hypothesis suggests that females from derived populations have a wider preference range than females of the ancestral population, allowing the range of the male trait to expand. Females from the ancestral population maintain a tighter preference and thus discriminate against males from the derived stock who display the trait outwith the ancestral females preferences range. However, the lack of female discrimination by 'ancestral' morph females against the 'derived' males in for morphs of 7,11HD (Ferveur *et al.*, 1996) agrees with the theory put forward by Kawanishi and Watanabe (1981), and does not agree with Kaneshiro's hypothesis of speciation (1976). It therefore seem unlikely that there is any one rule to the discrimination seen between ancestral and derived populations.

The cause of the change in IPI and in female preference is unclear, as well as any linkage between the two trends. It would seem that given the premating isolation, the existing nuclear differences shown by Begun and Aquadro (1993) could be maintained. The Zimbabwe strain is likely to remain genetically distinct from other strains of *D. melanogaster*. This could be, as Wu *et al* (1995) say in their paper's title, "A possible case of incipient speciation" demonstrating the importance that premating isolation plays in such events.

CHAPTER 6: HERITABILITY OF SONG AND MORPHOLOGY IN A NATURAL POPULATION OF *D. MELANOGASTER*

6.2 Introduction

In previous chapters I have examined IPI and female mating speeds with male strains differing in IPI. However, both female preference and itsmale trait need to be passed on to the next generation for sexual selection to have any effect on the evolution of these traits in subsequent generations. Inheritance is quantified by measuring the heritability and evolvability of the character being studied. Heritability of *D. melanogaster* is usually studied in the laboratory. Studying heritability in the laboratory reduces the level of environmental variation. This means that a larger proportion of the phenotypic variation is composed of genetic variation (V_G), thus possibly overestimating the heritability.

Strains that have been kept in the laboratory for many years may have lost much of the genetic variation found in the wild population from which they were derived through bottlenecks and adaptation to laboratory conditions. This will counter the overestimating effect that laboratory rearing has on genetic variation and heritability. However it is common to extrapolate the values obtained under laboratory conditions to natural conditions. In the field additive variation can be masked by large environmental effects, which can be several times larger than in the laboratory (Coyne and Beecham, 1987; Ruiz *et al.*, 1991). Differences between laboratory and natural environments may cause genotype-environment interactions that can inflate genetic

variances and bias estimates of heritabilities and genetic correlations (Riska *et al.* 1989; Hoffman, 1991).

Aspi and Hoikkala (1993) used father-son regression to study the heritability of four song characters of the two closely related species, *D. montana* and *D. littoralis*. *D. montana* had high and mostly significant heritabilities where both fathers and sons had been raised in the laboratory. Heritability values for *D. littoralis* were generally lower and not significantly larger than zero. They then used wild-caught fathers and laboratory reared sons for both species and found that most of the *D. montana* and all of the *D. littoralis* across-environment heritabilities were non-significant. This seemed to be due to the larger phenotypic variability in the field, and also genotype-environment interactions in some cases. Heritabilities were significantly smaller in some cases than those measured in laboratory conditions.

Roff and Mousseau (1987) and Mousseau and Roff (1987) found that life history traits generally possess lower heritabilities than morphological traits, with behavioural and physiological traits being intermediate. They showed that high heritabilities can be maintained in natural populations even when a trait is under strong selection. Fisher's fundamental theorem of natural selection states that "The rate of increase of fitness of any organism at any time is equal to its genetic variance in fitness at that time" (Fisher, 1930). This is generally thought to imply that traits that are closely and consistently associated with fitness will have low additive genetic variances due to natural selection. However it also assumes consistency of genotypic fitness, independence of genotypic frequencies, and population equilibrium. These may not be shown in natural populations.

Falconer and Mackay (1996) have concluded that traits closely connected to fitness possess low heritabilities, with those factors with high heritabilities having been subject to weaker or inconsistent natural selection. Their conclusion is derived largely from data from domestic animals that may be partially inbred. Factors that disrupt the assumptions of Fisher's Theorem could result in the maintenance of significant levels of genetic variation for high fitness traits in natural populations. These factors could play a part in populations of *D. melanogaster*. The species has been considered to be at equilibrium, with free gene flow across populations, that is a single global population (Singh and Rhomberg, 1987). However, this view has been recently challenged with the demonstration of a larger degree of population structure than previously thought and prezygotic barriers to exchange between populations (Begun and Aquadro, 1993; Wu *et al.*, 1995).

Two reasons for comparing genetic variation are to measure the ability to respond to selection, and to make inferences about the strength of selection (Houle, 1992). Genetic variation is usually compared using narrow sense heritabilities (h^2). Heritability within a population is the additive genetic variance (V_A) divided by the phenotypic variance (V_P).

$$h^2 = V_A/V_P$$

This will determine response to selection in the following generation (R), which is the product of heritability and selection differential (S).

$$R = h^2S$$

R is not a dimensionless quantity, and S also contains information about the selected population. By standardizing the mean of the population before selection both problems can be corrected for, and the coefficient of additive variation (CV_A) is more useful for comparing evolvability of traits than narrow sense heritability. The seemingly low heritability values of fitness components might be explained by a relatively low V_A, or by high residual (non additive) variance (CV_R), or both. The coefficient of residual variation (CV_R) is defined as $(100*\sqrt{(V_P-V_A)}/\text{mean})$. Coefficients of variation can be used to address the relative significance of V_A and V_R.

Houle (1992) reviewed over 200 studies and showed that traits closely related to fitness have higher additive genetic and non-genetic variability, as measured by the coefficient of variation, than traits under weak selection. This is the reverse of Falconer's conclusions from the comparisons of heritabilities. Houle explained this by a high residual variation. This high additive and residual variability may be due to the large number of genetic and environmental events that they are affected by, or by the lack of stabilizing selection. Pomiankowski and Møller (1995) suggest increased CV_A might be common in sexually selected traits despite, or even because of, directional selection.

Ritchie and Kyriacou (1994) found low heritability and evolvability of mean IPI in a European strain of *D. melanogaster* using father-son regressions. Despite this they achieved a substantial response to artificial selection resulting in a divergence of over 4 msecs in six generations (Ritchie and Kyriacou, 1996). The discrepancy between heritability and evolvability measures and the response to selection seen is surprising. It would be preferable to study wild populations for evolvability and heritability to determine if the results of Ritchie and Kyriacou are reflected in these

populations, and if their conclusions are applicable, and to examine if there is a discrepancy between the two scores. Due to the short lifespan of *D. melanogaster* it is difficult to measure IPI unless conducted at, or close to, the population site.

Here I have studied a wild population of *D. melanogaster* from Mauritius in the Indian Ocean. This population is a recent introduction from the African continent (David, 1981). Due to their short life cycle coupled with the transport time from Mauritius it was not possible to study wild-bred flies. However it was possible to study flies bred in the laboratory that were very recently derived from wild adults. I hoped that much of the genetic variation present in the wild population would be maintained compared to established laboratory stocks. This study meets the five criteria for estimates of evolvability, as set out by Houle in his review (1992). The criteria are described below.

i) Characters are assumed to be under strong directional, or weak stabilizing selection.

ii) The population is assumed to be near genetic equilibrium.

iii) The population is outbred.

iv) Additive genetic variance was estimated using relatively unbiased methods.

v) The variances are on an untransformed scale.

I wanted to measure IPI to establish the likely response to selection of a wild population and examine the validity of extrapolating results from laboratory stocks to wild populations. A newly collected stock from the same population that the artificial selection line was derived would have been ideal, but was unavailable. I also wanted to compare the values for the behavioural (song) traits with morphological traits, with reference to Roff and Mousseau (1987) and Mousseau and Roff (1987). If IPI is under strong selection I would predict lower heritability, though possibly high evolvability.

6.2 Methods

6.2.1. Stocks.

The stocks were collected by Dr. Rajabalee from the Mauritius Sugar Industry Research Institute, in March 1995. These had been collected by leaving 30 ml glass vials filled with medium in areas close to, and within, human habitations. Wild flies laid eggs into the medium and the vials were transported to the laboratory where all emerging flies were anaesthetised within twelve hours of emergence. *D. melanogaster* was identified using keys from Shorrocks (1972) and Ashburner (1989). All anaesthetisation of flies was by CO_2. Individual *D. melanogaster* were stored in separate vials with medium. Females were left for two days then the vials were inspected under the light microscope for eggs. Females that had not laid eggs were assumed to be virgin. Three males were placed in a fresh vial with medium with three virgin females, with replicate vials. The offspring were collected within twelve hours of eclosion and separated by sex, and stored in fresh vials for two days. The use of offspring was necessary due to the low number of adults emerging from wild-laid eggs.

Three minutes of courtship song of each of forty males was recorded (see section 6.2.2 for details). Then each male (sire) was placed in a vial containing medium with a single virgin female (dam A) for three days to allow fertilization and egg laying, then both flies were removed. The female was placed in a freezer, in a labelled eppendorf tube. The male was transferred to a fresh vial containing a second virgin female (dam B) and allowed another three days to mate. The male and female

were then removed and frozen. This meant that the flies died with the minimum damage to their bodies. They were then measured for morphological traits. This was replicated for forty sires and eighty dams, resulting in forty half sibling families and eighty families in total.

The offspring from each tube were examined on the day of eclosion, and five males from each family were placed in separate fresh vials for two days. The courtship song of each male was recorded and analysed. The flies were killed and measured as before. Five males from each family were recorded, making ten sons from each sire in total.

Father-son and half-sib regressions were carried out on both interpulse interval (IPI) and intrapulse frequency (IPF). Father-son, mid-parent-son, and half-sib regressions were carried out for all morphological traits.

In this study, a total of 40 sires was measured with 80 dams and 400 sons. The total number measured was therefore 520. Hill and Nicholas (1974) state that for a heritability variance of 0.01, 20 sire families with a total of at least 500 observations over two generations are needed.

6.2.2 Song analysis.

Single males were isolated within twenty four hours of eclosion and placed into 30 ml glass vials containing medium. After two days the vial was placed in the microphone chamber of the insectavox (see chapter 2 for full description) for at least one minute in order for their body temperature to equilibrate. The male was aspirated into the courtship chamber, without anaesthetic, with a virgin female. The female had been collected and muted on the day of recording, and within 24 hours of eclosion. Recording was started once the male had produced one burst of pulse song, and continued for the next three minutes of courtship. All recordings were carried out during the twelve hour light period of the diurnal cycle Fluctuations in temperature were limited using external heaters and fans and held close to 25°C.

The songs were sampled directly into an IBM compatible computer after being filtered (high pass 250 Hz, low pass 1 KHz), and digitized by a 1401 analogue-digital converter (sample rate of 4 MHz). The recording was written as a SPIKE2 formatted file.

The mean IPI was calculated using SPIKE2 (C.E.D.) software, by a prewritten analysis program FASTDMEL.TXT. Manual editing of events was carried out using EVENT.TXT (for description see chapter 2). The intrapulse frequency (IPF) was calculated using the analysis program FLYFFT.TXT. For each song recording a pulse burst of high amplitude was selected to minimise the effect of background frequency. A fast fourier transfer was automatically performed on this area, which resulted in a power spectrum of the pulse burst over the frequency range of zero to 1000Hz. The frequency at the highest point of the spectrum was measured. One IPF measure was

taken for each male. This character does not influence female preference (Bennet-Clark and Ewing, 1969).

Mean IPI and IPF were regressed against temperature using the data from males of both generations. The regression coefficients were tested for significance. The mean IPI was adjusted to 25°C. The IPF was not significantly related to temperature and no adjustment was made.

6.2.3 Morphological measurements

Four morphological traits were measured; Thorax length, wing length, foreleg femur length and sternopleural bristle (or chaeta) number. All measurements were carried out under a light microscope at X40 magnification. All lengths were measured using a 1 cm graticule with 100 gradations. Only one side of each individual was measured with no systematic control over which side was used.

Thorax length was defined as the measurement from the dorso-anterior corner of the mesopleuron to the posterior edge of the scutellar margin. Femur length was defined as the length from the joint with the trochanter to the joint with the tibia, as shown in figure 6.1. Wing length was defined as a straight line along the third longitudinal vein, from linkage with the anterior cross-vein to linkage with the costal vein, (shown in figure 6.2).

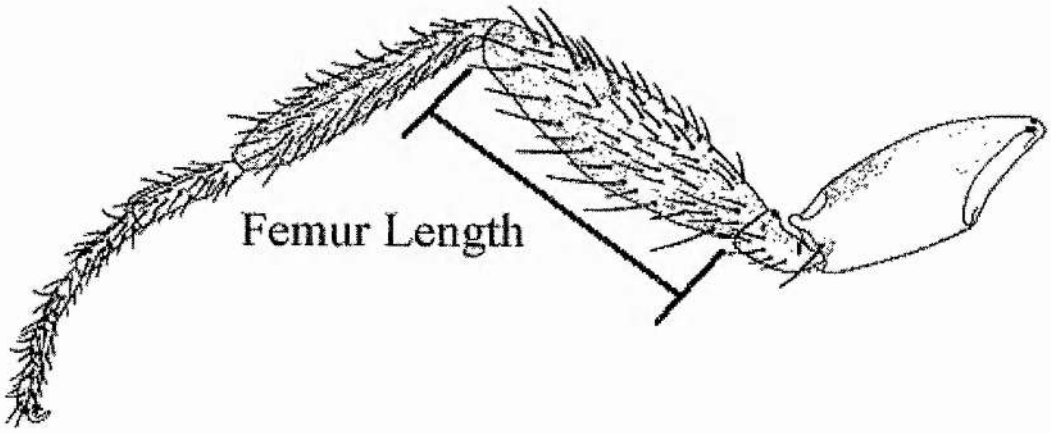


Figure 6.1 shows the femur length measured on the foreleg.

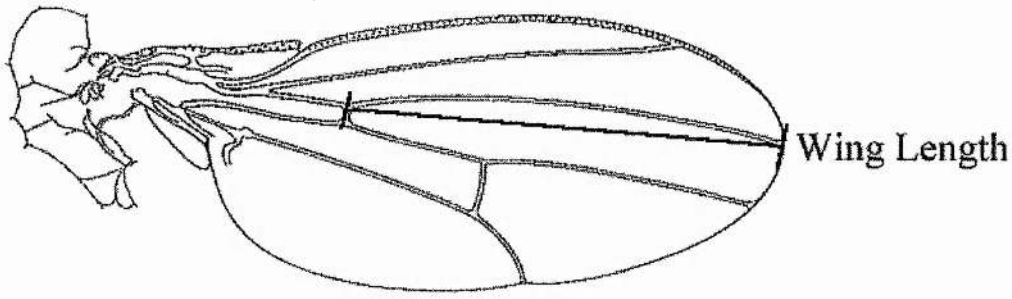


Figure 6.2 shows the length measured on the wing.

Thorax length, wing length and femur length can be used as estimates of total body size. It has been thought that body size is important in inter-male competition and sexual selection (Partridge *et al.* 1987). Such traits are assumed to be under strong directional selection (Roff and Mousseau, 1987). Values previously obtained for heritability of thorax length from a field population are 0.357±0.03 (Tantawy and El-Helw, 1970), and 0.344±0.024 (coefficient of variation 2.62) (Tantawy and Tayel, 1970). Both estimates are derived from parent-offspring regressions. Wing length

heritability from a field population has been estimated from parent-offspring regression as 0.362±0.032 (coefficient of variation 2.46) (Tantawy and Tayel, 1970). In both experiments twenty pairs of parents were measured with twenty offspring of each pair.

Sternopleural bristle number was used as the character is known to be under weak stabilizing selection. Thoday (1958) claimed that sternopleural bristle number was adaptive as it varied between populations. Reeve (1960) suggested that genes controlling the trait also control another trait under selection. Kearsey and Barnes (1970) demonstrated that the pleiotropic effects on the trait were under stabilising selection in the laboratory, and selection on characters pleiotropically regulating the trait acts at the larval stage before sternopleural bristles develop. Sheridan *et al.* (1968) measured father-son heritability at 0.17±0.08, and mother-son heritability at 0.33±0.09, with the pooled heritability value being 0.31. Measurements were taken across 62 sires, with three dams per sire, and 10 males per dam. All measurements were carried out on a field population.

6.2.4 Analysis of heritability values

Father-son heritability, evolvability and residual variance values were calculated for both song traits and the four morphological traits using the methods described by Falconer and Mackay (1996). The previous estimations of heritability in all studies mentioned, including the artificial selection experiment for long and short mean IPI (chapter 3, and Ritchie and Kyriacou, 1994), follow the general form set out by Falconer in previous editions of Falconer and Mackay (1996). The method of analysis is summarised below.

The mean for each trait was calculated from the measurements of the trait in all sires, and the phenotypic variance of each also noted. The means of all values from the sons of each sire were calculated. The mean values were regressed to the values of the sires. As the value derived represents only half the additive genetic variance of the parents the result is doubled to obtain the h^2 value.

Additive variance was derived from $h^2 = V_A/V_P$, and calculated as $V_A = h^2 * V_P$. Evolvability was then calculated as the coefficient of additive variation ($CV_A = 100\sqrt{V_A}/\text{mean}$). The coefficient of residual variation, ($CV_R = 100*\sqrt{(V_P - V_A/\text{mean})}$), could also be calculated once V_A was determined.

Mother-son heritability values were calculated in the same manner as the father-son values. The mean values for sons from each family were regressed with the value for the dam of each family. The data derived from families A and B were combined in one set. The regression value was then doubled to obtain the heritability value.

Mid-parent-son heritability values were also calculated in the same manner. The mean values for sons from each family were regressed with the mean value of the sire and dam of each family. The data derived from families A and B were combined. The regression value was then doubled to obtain the heritability value as the covariance of the offspring with the mean of both parents is the same as the covariance with a single parent.

The mean values for each trait in sons for each family were calculated. A correlation of the mean values from sons of different families with the same sire, i.e. family A and B, was performed. The half-sib heritability value was determined to be four times the correlation value. Half-sibs have the same sire but a different mother. Covariance is half the breeding value of the common parent, being a quarter of the additive variance. All regressions and correlations were performed using the MINITAB program.

Sheridan *et al.* (1968) noted the poor agreement between offspring-parent and sib-covariance heritability values in sternopleural bristle number, and thought that this was due to sampling error. However, it is commonly assumed that various sources of common environmental variance do not contribute to the parent-offspring heritability estimates. Sources of common environmental variance would contribute to heritability estimates from half-sib correlations, hence giving a higher value for the latter. Hill and Nicholas (1974) argue that pooled estimators based on both between offspring-parent and sib-covariance heritability values are better than the use of either regression or sib-covariance alone. Therefore all available estimates were pooled for each trait.

Pooled heritability values were calculated as the mean of the father-son regression and half-sib heritability values, while the pooled values for morphological

traits were calculated as the mean of mean of the father-son regression, mother son regression, midparent-son regression and half-sib heritability values.

Negative heritability values were treated as being effectively zero. It is deemed inappropriate to calculate the evolvability value from negative heritability scores, and both heritability and evolvability are taken as being effectively zero.

Trait	Father-son h^2	Half-sib h^2	Mother-Son h^2	Midparent-son h^2	Pooled h^2	Evolvability[(1)]	Evolvability[(2)]	CV_R
Intrapulse Frequency	0.062±0.066	0.672±0.518	-	-	0.367	4.765	11.613	374.135
Interpulse Interval	0.206±0.194	-0.508±0.521	-	-	-0.151	3.742		47.286
Sternopleural Bristles	-0.105±0.184	0.284±0.524	0.288±0.169	0.476±0.259	0.235	-	7.307	33.031
Wing Length	0.251±0.178	0.78±0.515	0.204±0.158	0.8±0.226	0.508	4.156	5.908	8.107
Femur Length	-0.069±0.134	-0.176±0.525	0.028±0.066	0.252±0.192	0.009	-	0.915	6.538
Thorax Length	0.171±0.15	0.233±0.511	0.228±0.098	0.372±0.162	0.251	3.920	4.743	7.200

Table 6.1 Heritability, evolvability and residual variance values of all traits measured. (± Standard error values).

[-] = no data; [Evolvability[(1)]] = Evolvability from father-son heritability; [Evolvability[(2)]] = Evolvability from pooled data.

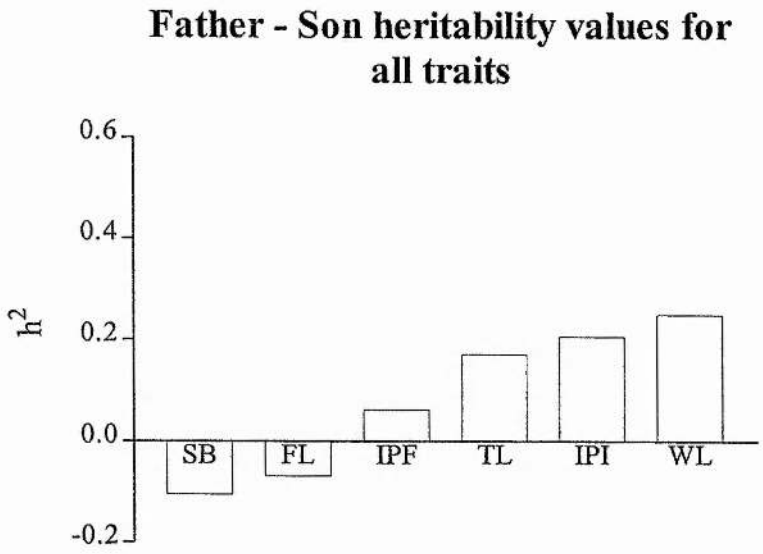


Figure 6.3 Heritability values for all traits from father son regression values. (IPF = wing length; IPI = Interpulse interval; SB = Sternopleural bristles; FL = Femur length; TL = Thorax length; WL = Wing length)

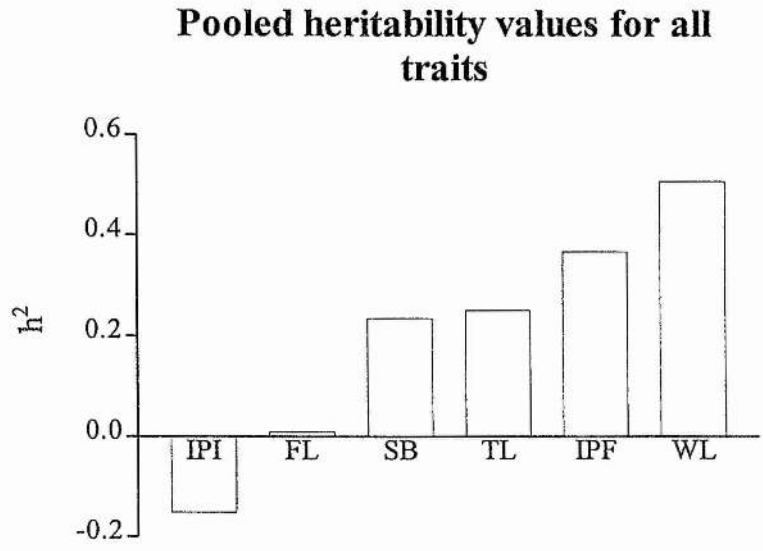


Figure 6.4 Pooled heritability values for all traits.

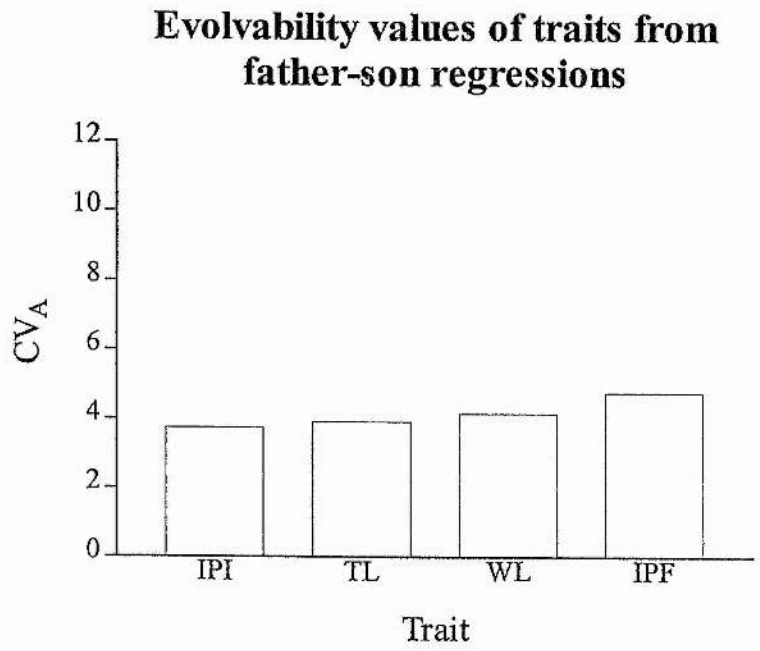


Figure 6.5 Evolvability (CV_A) values for all traits derived from the father-son values for heritability.

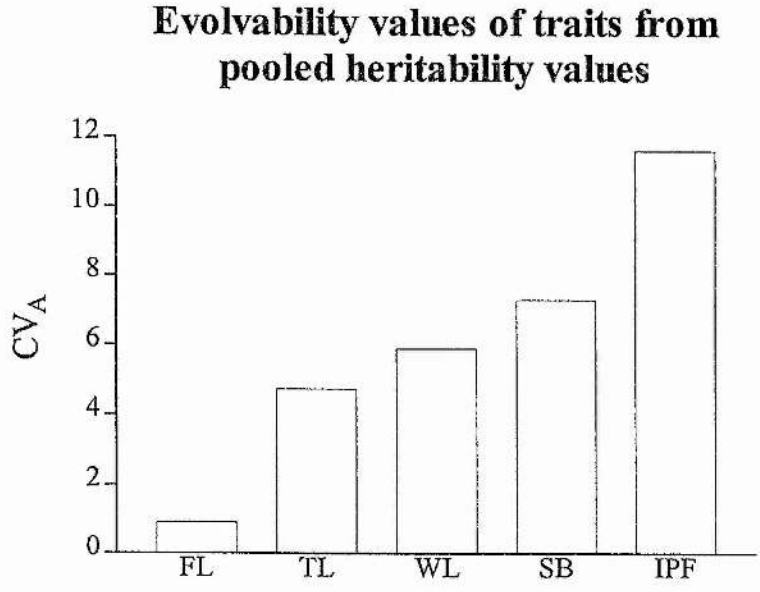


Figure 6.6 Evolvability values derived from pooled heritability values.

6.3 Results

Heritability values for all traits are show in table 6.1, as well as the coefficient of additive variation (CV_A), or evolvability, and the coefficient of residual variation (CV_R). Figure 6.3 Shows the average heritability scores from the father son regression of both families. Figure 6.4, shows the average heritability from pooled heritability measures. Figure 6.5 shows the evolvability of each trait from father-son regressions while figure 6.6 shows the evolvability derived from the pooled heritability values.

Most heritability values are not significantly different from zero, so the relative relationship between heritability values for each trait should not be weighted too strongly. The heritability values obtained from the father-son regressions and half-sib correlations is highly divergent in most cases. It would therefore seem likely that there may be a large degree of error due to measurement error, or some other factor. However, measurement error was not estimated.

All evolvability values are low, and there is a wide variation in the heritability scores for each trait depending on the method used. The midparent-son heritability values are significant for all morphological traits except for sternopleural bristle number. This is likely to be a reflection of the heritability value being derived from the covariance of both parents instead of just one. Midparent-son heritabilities are also the highest, suggesting that the other measures have underestimated the true value of heritability for each trait. The pooled heritability and evolvability values were felt to be the most accurate measure of the true population heritability level, as stated by Hill and Nicholas (1974).

IPF is not thought to be an important factor of courtship song and shows the largest pooled heritability value, although no measures for the trait were significant.

The residual variation is by far the highest of all traits measured, being a factor of ten higher than that of the other values. The high residual variation also means that IPF has the highest evolvability values of all traits. The father-son heritability value was very close to zero, with the large pooled heritability being mainly due to the large half-sib value.

The pooled heritability value for IPI is negative, being treated as being effectively zero, and no evolvability value was calculated. The pooled evolvability was not calculated. IPI has a high residual variation. Both the heritability and evolvability values from the father-son regression are similar to that obtained by Ritchie and Kyriacou (1996). The large negative heritability value of the half-sib correlation contradicts the result from the father-son regression, with the former suggesting a much higher potential response to selection.

Sternopleural bristle number has a low pooled heritability. However the evolvability is higher than other morphological traits with larger heritabilities. This is due to the trait having the highest residual variation of all morphological traits. Father-son heritability is negative, with all heritability measures being non-significant. Evolvability for the trait suggests more rapid respond to selection than its heritability. The values from parent-offspring regressions broadly agree with Beardmore *et al.* (1975), although the half-sib value is lower.

The pooled heritability value for femur length is very low. The pooled evolvability is the smallest of all traits measured. The low value is a result of the low residual variance as well as low heritability. Both father-son and half-sib heritability values are negative, which is probably due to experimental error. The true heritability value is however, most likely to be extremely low as the pooled heritability value suggests. Femur length is likely to show very little response to selection, in line with a

trait that has been under strong directional selection as would be expected for a trait closely linked with overall body length. The midparent-son heritability value is significant ($F_{1,62}$=4.5, P<0.05), and would suggest that there may still be some degree of response to selection.

Wing length has a high pooled heritability value at just over 0.5. However, evolvability suggests a more modest response to selection, due to low residual variance. The mid-parent son value is very high (0.8), and highly significant ($F_{1,58}$=12.46, P=0.001). Although all other heritability measures are smaller, none is significant. It would still be reasonable to suggest that wing length has a higher heritability than both femur and thorax length. The high heritability for wing length may be due to the trait having been under less strong directional selection than these other two traits, due to stronger stabilizing selection pressures because of the wing's importance in flight. The heritability value is higher than those recorded by Tantawy and Tayel (1970), however separate heritability measures cover a large range of about 0.6, with two low and two high values.

Thorax length pooled heritability and evolvability values fall between femur length and wing length. Both the mid-parent-son ($F_{1,62}$=5.22, P<0.05) and mother-son ($F_{1,65}$=5.46, P<0.05) regressions are significant, and all heritability measures are similar. It is therefore likely that the heritability and evolvability values of this trait represent the most accurate measure of all traits. Again this trait is likely to be under indirect selection for body length and mass. The parent-offspring heritability values are similar to Tantawy and El-Helw (1970), and Tantawy and Tayel (1970).

6.4 Discussion

The disparity between heritability and evolvability emphasises the importance of including evolvability values in the examination of genetic variability and response to selection. This finding is in essence, the underlying point made by Houle (1992). The most notable example from this study was evolvability obtained from the father-son heritability value. Evolvability implies the trait would respond to selection at a higher rate than the heritability value would suggest.

The values for heritability and evolvability for IPI found here are similar to those for the population measured by Ritchie and Kyriacou (1996). It would seem that results from laboratory stocks could reasonably be extrapolated to wild populations. Further work needs to be done to confirm these findings. Given the same magnitude of selective pressure in nature, the resultant effect on IPI is likely to be similar to that seen in chapter 3. The fact that change in IPI is possible but largely unseen in the field points to broadly stabilizing selection in natural population. Directional sexual selection for short IPI is suggested to occur by Ritchie and Kyriacou (1996), and from the results of female mating speeds in different strains of *D. melanogaster* chapter 4, and chapter 5. Stabilising selection may be due to natural selection countering this possible directional sexual selection.

Ritchie and Kyriacou (1994) stated that IPI has been under strong selection, and is unlikely to respond rapidly to selection. The values for heritability and for evolvability indicate a little potential to respond to selection, suggesting that IPI is a trait that may have been under strong sexual selection. This contrasts with Cowling (1980) who states that IPI had a high heritability, and a strong response to artificial selection has been shown (Ritchie and Kyriacou, 1996; chapter 3). Given this

disparity between heritability measures and response to selection a clear picture does not emerge from this previous work. The response to artificial selection broadly agrees with Pomiankowski and Møller (1995) who state that polygyny can be maintained even in traits under strong sexual selection.

Evolvability values are not reported by Aspi and Hoikkala (1993) in their study of natural populations of *Drosophila montana* and *D. littoralis*. However, the lack of significant heritability values is similar to the values for song traits in the present experiment.

As there isonly a total of six traits measured it is only possible to say that the results are consistent with the findings of Roff and Mousseau (1987). There is broad agreement of the heritability estimates for traits measured in previous studies (Tantawy and El-Helw, 1970; Tantawy and Tayel, 1970; Beardmore *et al.*, 1975). However, the wide degree of variance of most heritability measures in this study mean that agreement is almost certain.

This study assumes that the population measured is at equilibrium. However, it comes from a population which has been recently established on the island of Mauritius. The island also contains the sibling species *D. mauritiana*. There may have been only a small founder population situated around human habitation, and some degree of competition for resources between the two species and therefore the *D. melanogaster* population may not be at equilibrium.

Forty sire families and a total number of 520 individuals were measured, more than the number suggested Hill and Nicholas (1974). The large standard errors seen here are therefore surprising. It may be that although I had enough observations in total, more offspring per family were needed. The other possibility is that of experimental error. This could come about though errors in length measurements of

morphological traits. This does not seem to be a viable explanation for measures of sternopleural bristle number or the two song traits. It may have been better to perform measurements on both sides of the body. The effect of background noise and harmonic frequencies may have resulted in incorrect IPF values. Several measures for each song might have reduced this problem.

The large variations in different heritability estimates for each trait, coupled with few of the values being significant makes interpretation of the results difficult. Adults from each generation were recorded over two days to reduce the environmental effects on IPI between separate recording sessions. This reduced the total number of individuals that could be measured. Expanding the study to include more individuals could have only been done by pooling the results from additional, separate experiments, which suffers from between session variance in IPI. IPI was shown to be at least as low as the morphological traits measured.

Enormous sample sizes are required for accurate heritability measures. It is therefore ambitious to obtain enough recordings. Although the numbers used were possibly not large enough, some significant heritability measures were seen. However given the large number of comparisons carried out here, there is the real possibility that heritabilities that have been found to be significant may be an statistical artefact. Although there are tests to account for multiple comparisons (e.g. the sequential Bonferroni correction index) these were not used, and would be needed to rigourously test the significant measures seen.

CHAPTER 7: GENERAL SUMMARY

This research has illustrated the importance that IPI plays in female discrimination and sexual isolation in the *D. melanogaster* complex. The role that pheromones play in reproductive isolation among species of the *melanogaster* complex has also been examined recently, most notably by Coyne and co-workers (Coyne *et al.*, 1994; Coyne, 1996a; 1996b). There have been several papers published showing that single chromosomes play a significant effect on cuticular hydrocarbon balance, which is also important in sexual isolation within *D. melanogaster* (Scott, 1988; 1994; Ferveur and Jallon, 1996; Ferveur *et al.,* 1996). The same single chromosome effect has also been seen in these interspecies studies. It might therefore be perceived that the role which IPI plays in sexual isolation and speciation is secondary. However, differences in IPI may be important in sexual isolation between the Zimbabwe strain and other strains of *D. melanogaster* (chapter 5). Pheromone blend affects the male recognition of females, while it is IPI that affects male discrimination in females. Therefore sexual selection and isolation in *Drosophila* is most likely to be controlled by both pheromone blend and IPI.

The Zimbabwe strain is of great interest as it is the first strain to show sexual isolation in a species that was formally thought to possess no isolation between populations throughout its geographical distribution (Henderson and Lambert, 1982). The potential for research into the role of sexual isolation in speciation, which has been so intensively studied and characterized, is great. The lack of postmating barriers opens many avenues of research closed to similar studies between species. The production of fully viable and fertile offspring allows the use of crossing protocols

which are impossible to use when one sex is infertile, as is the case for *D. simulans/D. mauritiana* crosses.

Examination of the pheromone blend of the Zimbabwe strain has found no differences from the other strains with which it shows sexual isolation (H. Hollocher, Univ. Chicago, personal communication). The difference in IPI of males from the Zimbabwe strain has not been causally linked with female preference. However, given the lack of pheromone difference, it seems likely that the females discriminate against males from other strains on the basis of IPI.

The significant effect that one chromosome plays in the control of pheromone blend between *D. mauritiana* and *D. simulans* has been demonstrated (Coyne *et al.*, 1994). In contrast, the control of the difference in mean IPI between the same two species was shown to be evenly spread over the five separate regions of the genome (chapter 2). This disparity highlights the fact that changes responsible for sexual isolation between two populations may not necessarily have a similar genetic control. It is, as yet, impossible to quantify fully the number of genes causing the difference between species, and their relative contribution, for both differences in IPI and in pheromone blend. The large effect that the third chromosome plays in pheromone blend may be the result of a single gene, or several linked loci. The relative effect of linkage is likely to be much greater than for IPI for which the controlling genes are more widely spread thoughout the genome.

The difference in mean IPI between *D. simulans* and *D. mauritiana* was shown to be under autosomal additive control in the analysis reported in chapter 2. From the results of artificial selection (Ritchie and Kyriacou, 1996), this would also seem to be the case for the difference in IPI within *D. melanogaster*. The results of the chapters 2

and 3 taken together suggest that the trait is under similar genetic control thoughout all species within the *D. melanogaster* complex.

Hall (1994) examined the effect of mutations in single genes on courtship song. Single genes have been shown to affect the length of the song cycle (*period*), receptivity of females (*spinster*), and the cycle number of individual pulses (*cacophony*). These examples show that changes of single genes could affect the nature of the courtship and courtship song. The process of isolating the genes that control IPI will be complicated if there are no single genes of major effect (Cowling and Burnet, 1981; Ritchie and Kyriacou, 1994; Chapter 3). It is possible that there is a single gene controlling pheromone balance, which could be isolated and characterized, as with the *period* gene. Characterizing single genes provides a more attractive end result than defining the relative effects of different areas of the genome. However, Liu *et al.* (1996) have shown, through QTL analysis, that the relative contributions of sections of the genome can be studied in great detail. Their study resulted in the identification of QTL effects of only between 5 and 16% of parental differences.

The results of chapter 2 have already been published (Pugh and Ritchie, 1996)and the findings have formed the basis for a successful grant application. The forthcoming study plans to use molecular markers to examine the genetic control of the difference in IPI in further detail. Future work on the selection lines produced in the study reported in chapter 3 could involve a similar analysis of the genetic control of IPI, using isogenic lines. Such a study would benefit greatly from the wealth of markers available within the species and the lack of hybrid inviability between stocks seen in inter-species studies.

In chapter 4 of this thesis females of the Pietrastornina strain mated significantly faster with males from a short selection line than with males from the long selection line. A similar trend was seen with females from lines from both the Short and Long regimes. Females from the Zimbabwe strain measured in chapter 5 also mated more readily with males possessing a relatively short IPI. Females from a French strain, in which males possessed a relatively long IPI, did not display a discernible difference in mating speed between males from their own strain and males from the Zimbabwe strain. It may be that in most populations of *D. melanogaster,* females mate more readily with (i.e. prefer) males with a relatively short mean IPI. However, evidence of such a female preference remains elusive.

Although females from which all selection lines were derived mated faster with males from the short selection line, mating between males and females was arbitrary during selection, with males placed with random sibling females. Arbitrary matings would not have allowed the maintenance of genetic covariance of the distribution of male trait and female preference (Pomiankowski and Sheridan, 1994). The fact that females from the selection lines do not seem to discriminate between potential mates according to their IPI, suggests that discrimination has been lost during the course of the selection experiment. Matings were not arbitrary in the Zimbabwe strain used in chapter 5. Therefore one can assume that the female mating speeds of the Zimbabwe strain are a close representation of the female preference seen in the natural population. Females from the Zimbabwe strain tended to prefer males with similar IPI over males from the France control strain. The IPI of males from the Zimbabwe strain (≈29 msecs) was significantly lower than the mean IPI measured in other strains. The preference of Zimbabwe females for males of their own line was therefore probably due to covariance of female preference for IPI with IPI.

The discovery of the Zimbabwe strain should generate renewed interest in IPI, and its importance to sexual isolation in the *D. melanogaster* complex. The study of the difference of the mean IPI of the Zimbabwe strain is only at an initial stage. The genetic control of the difference in IPI of the Zimbabwe strain with other *D. melanogaster* has not been examined. A study would be needed to determine if the difference is under a similar control to the difference between *D. mauritiana* and *D. simulans*. Such a study will again benefit from advantages of full viability between strains and the large number of potential QTL markers already available, as compared with a similar interspecies study. However, unlike during artificial selection, females have been allowed to choose their mates in the Zimbabwe stocks. It would therefore be possible to investigate covariance of female preference for IPI, and IPI. Choice studies of female preference in the Zimbabwe strain would be preferable to the no-choice trails used, due the unusually low levels of mating seen by the Zimbabwe females with all male lines reported in chapter 5. Choice trials can also measure relative mating success between males from different lines. This is not possible to measure in no choice tests.

Few of the heritability measures reported in chapter 6 were significant. However, heritability and evolvability within this population were found to be similar to that seen in the laboratory strains (Ritchie and Kyriacou, 1994; Chapter 3). The strong response to artificial selection that has been shown (Ritchie and Kyriacou, 1996; Chapter 3), is therefore somewhat surprising. The apparent paradox of a population with low heritability showing strong response to selection is not fully resolved by the results in chapter 6. The heritability results would seem to confirm that IPI will not show a large response to selection. However, the evolvability values suggest more genetic viability than the narrow sense heritability values do. The

evolvability values may hint at how such a large response may have been possible. Narrow sense heritability measures may be confounded by the residual variance (V_R) and by scaling factors, which are taken into account in evolvability measures.

Changes between populations come about due to adaptation to the different environments that the populations occupy, and also due to drift. It may therefore be interesting to study how courtship and mating are affected by adaptation to different environmental factors such as temperature. For instance, cuticular hydrocarbons are known to play a role in protection of the cuticle against desiccation (Edney, 1977). The relaxation of environmental pressure in a new environment that had previously limited the range of a male signal, would result in an increase in variance of the trait. If female preference was directional then sexual selection could shift the distribution of the trait without the countering effect of natural selection.

Differences in IPI within the *D. melanogaster* complex play an important role in sexual discrimination and premating isolation, both within *D. melanogaster* and between species. The use of computer technology to analyse recordings, and advances in QTL mapping using molecular markers, means that future research in this area should yield both interesting and detailed results. The study of IPI remains important in furthering the general understanding of the genetic control of premating isolation between taxa of *Drosophila* that contributes to speciation, and of traits important in sexual selection.

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